Root colonization and environmental fate of the bioherbicide *Pseudomonas fluorescens* BRG100

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Food and Bioproduct Sciences, University of Saskatchewan

by:

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Abstract

Pseudomonas fluorescens BRG100 produces secondary metabolites with herbicidal activity to the grass weeds wild oat, *Avena fatua*, and green foxtail, *Setaria viridis*. The green fluorescence protein (gfp) gene was introduced into *P. fluorescens* BRG100 from *Escherichia coli* S17-1λ via a Tn5 mini transposon suicide vector system. Colony morphology, growth rate in liquid media, weed biocontrol efficacy (plant growth pouch), carbon utilization (Biolog GN) and root colonization of green foxtail by several *P. fluorescens* BRG100*gfp* transformants were determined to be the same as the wild type. *Pseudomonas fluorescens* BRG*gfp*-15 was found to be most similar to the wild-type in all of the above characteristics and was thus used in subsequent experiments. Note: all strains of *Pseudomonas fluorescens* will be referred to by only their strain throughout (ie. BRG*gfp*-15 and BRG100).

It was determined by population dynamics per section of root with spiral plating on culture medium, epi-fluorescence and confocal microscopy that BRG*gfp*-15 colonized all areas of the root, but showed a preference for the proximal 1/3 section and the seed. In the proximal section the mean number of viable cells per gram dry weight was $\log_{10}9.06$ and $\log_{10}9.31$, when applied as liquid inoculum and as the pesta granular formulation, respectively. With liquid inoculum there was only $\log_{10}7.53$ viable cells/g in the middle 1/3 section and $\log_{10}7.01$ viable cells/g in the distal 1/3 section. The number of viable cells/g with pesta granules was $\log_{10}7.61$ and $\log_{10}7.34$, for the middle and distal sections, respectively. The root hairs, root tip, and ventral portion of the seed were all areas of heavy colonization relative to the other areas of the root.

Survival of BRG*gfp*-15 in the pesta formulation was examined in 2 soil types, clay and clay loam, in a thermogradient plate apparatus by a factorial randomized design complete block experiment. The experiment included: 3-12 hour diurnal temperature regimes: 5-15ºC, 15-25ºC, and 25-35ºC and 3 moisture levels: 25, 50 and 75% of soil moisture holding capacity. Sampling was carried out after 0, 14, 28 and 42 days. The highest numbers of viable BRG*gfp*-15 cells/g were found in the pesta granules in soil subjected to the lowest diurnal temperature regime and moisture content. The lowest numbers of viable cells/g were found in the pesta granules incubated in the highest diurnal temperature and moisture. This suggests lower soil temperature and moisture enhances survival of BRG*gfp*-15 in pesta and/ or higher soil temperature and moisture enhances the release and dissemination of BRG*gfp*-15 from pesta granules. When subjected to a 5-15ºC-temperature regime the number of viable cells/g was $\log_{10}9.80$. When subjected to 15-25ºC the viable cells/g was $\log_{10}8.96$ and with 25-35ºC it was $\log_{10}7.33$. The mean number of viable cells/g was $\log_{10}9.36$, $\log_{10}8.86$, and $\log_{10}7.87$, for 25, 50, and 75% soil moisture holding capacity, respectively. There was also a significantly higher number of viable cells/g in the clay soil collected from Saskatoon, $\log_{10}9.00$, as compared to the clay loam soil collected from Scott, which was $\log_{10}8.40$.

These results suggest that *Pseudomonas fluorescens* BRG100 has considerable potential as a bioherbicide because of its successful root colonization of green foxtail and wheat. *Pseudomonas fluorescens* BRG*gfp*-15 survived well under various environmental conditions when formulated into pesta granules, proving the pesta formulation was an excellent formulation. In addition, gfp was shown to be an excellent conservative marker for monitoring the root colonization and survival of *P. fluorescens* BRG100.

Acknowledgements

I would like to express my gratitude to all those who helped in the completion of my thesis. Thank you to my supervisors Dr. Susan Boyetchko and Dr. Darren Korber, as well as Dr. Russell Hynes, Dr. Phyllis Shand, and Dr. Vladimir Vujanovic, the members of my advisory committee. I would like to thank Jon Geissler for contributing a great deal of guidance and time over the course of my project, especially during my thermogradient plate assay, as well as Ross Weiss for his careful watch over the operation of the thermogradient plate apparatus. Thanks are expressed to Karen Sawchyn and Dan Hupka for contributing their time and lab space many times during the completion of my research. I would also like to thank Dr. Lorraine Braun for allowing me to use the epifluorescent microscope in her lab. Thanks are expressed to Dr. John R. Lawrence of the National Water Research Institute for the use of their confocal microscope and to George Swerhone for his aid in taking the pictures with the confocal microscope.

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1 Introduction

1.1 Rationale

Weeds cause significant yield loss in economically important crops such as corn soybean, cereals, canola, sugar beet, and pastures. Chemical herbicides have long been used to control weed growth, however several concerns have been raised about some chemical herbicides with undesirable environmental and health risks (Johansen and Olsson, 2005). Biological control (biocontrol) of weeds with microorganisms provides a useful alternative to chemical herbicides and several biocontrol agents have already been either registered or given permission for use around the world (Boyetchko, 2005).

Research at the Agriculture and Agri-Food Canada (AAFC) Saskatoon Research Centre has been directed towards developing bacteria as biocontrol agents of grass weeds in a pesta granular formulation. The pesta granular formulation is an extruded product composed of a cereal grain flour and any microbial biocontrol agent. *Pseudomonas fluorescens* BRG100 is a pathogen of the weed green foxtail (*Setaria viridis*) (Daigle *et al.,* 2002). *Pseudomonas fluorescens* BRG100 was isolated from a green foxtail seedling during a seed germination assay at AAFC in 1995 and has since been proven to be highly-effective in suppressing the growth of green foxtail in laboratory and field trials. In a field trial in 2000 the suppression of green foxtail emergence was as high as 83% when BRG100 was formulated into pesta granules (Daigle *et al*., 2002).

1.2 Purpose of Study

Pseudomonas fluorescens BRG100 has potential and may eventually be registered and commercialized as a bioherbicide for the control of grass weeds. In order for a bacterium to gain registration approval as a biocontrol agent, certain knowledge must be acquired. Examining the root colonization patterns of a bacterium can aid in the understanding of the spatial-temporal interactions between the bacterium and the target weed. Furthermore, it is important to reveal the effect that soil temperature, moisture, and texture will have on the establishment, colonization, and survival of the bacterium. This is useful for improving and predicting the efficacy of the biocontrol product under varying environmental conditions.

1.3 Objectives

The objectives of this study were to:

1. Develop a *gfp*-marked strain of *Pseudomonas fluorescens* BRG100 for monitoring colonization of green foxtail roots and survival in soil.

Pseudomonas fluorescens BRG100 was transformed with green fluorescent protein (gfp). Sixteen transformants were isolated based on the intensity of the gfp when induced. The transformants were compared to the wild-type for colony morphology, biocontrol efficacy of green foxtail, carbon utilization, and growth in standard media. The resultant strain of BRG100 expressing the *gfp* gene with the greatest similarity to the wild-type was used in subsequent experiments.

2. Evaluate root colonization of *P. fluorescens* BRG*gfp*-15 using dilution plating and confocal and fluorescence microscopy.

Efficacy of *P. fluorescens* BRG100 *gfp* on green foxtail in a liquid culture and as pesta granules was examined. Localization of *P. fluorescens* BRG*gfp*-15 on green foxtail roots was determined by confocal and fluorescence microscopy. Population dynamics of BRG*gfp*-15 was determined on the proximal, middle and distal sections of the green foxtail root by serial dilution and plating onto selection media.

3. Assess survival of *P. fluorescens* BRG*gfp* in soil under various environmental conditions.

Soils (0-20cm) from Saskatoon and Scott research farms representing different soil types and textures were collected and chemically characterized. Soils inoculated with pesta granules containing *P. fluorescens* BRG*gfp* were incubated at various moistures and temperatures in the thermogradient plate apparatus to assess the effect of these environmental conditions on survival of *P. fluorescens* BRG*gfp*.

1.4 Hypotheses

1. *Pseudomonas fluorescens* BRG*gfp*-15 will colonize certain areas of green foxtail roots more heavily than other areas of the root.

There will be significant differences between the populations on the three lengthwise sections of the root. *Pseudomonas fluorescens* BRG*gfp*-15 will also preferentially colonize certain areas of the root known to be regions of high exudation.

2. The survival of BRG*gfp*-15 will be significantly affected by the different environmental conditions.

The different soil temperatures, moistures, and types will affect the survival or dispersion of *P. fluorescens* BRG*gfp*-15.

2 Literature Review

2.1 Biological control

Biological control (biocontrol) is the use of organisms to subdue the effect of a specific pest, reducing the extent of its damage (Eilenberg, 2006). Biocontrol agents can be used for the control of detrimental insects, plant diseases, and weed plants. There are three different types of biocontrol: classical, inoculation, and inundation. Classical biocontrol is the intentional introduction of a foreign biocontrol agent intended to establish itself for long-term pest control. Inoculation biocontrol is similar in that it is the intentional release of a biocontrol agent, but it is expected to control the pest for a limited period of time, and thus is not permanent. It also differs in that it commonly involves non-foreign organisms as the biocontrol agent. Inundative biocontrol involves the application of the control organism after a pest population has increased to a certain point, even once the economic injury level has been surpassed. This requires a large amount of the biocontrol agent to be applied (Eilenberg, 2006).

Weeds, fungal and bacterial plant pathogens, as well as invertebrate pests can be suppressed with the intentional application of large amounts of the biocontrol agent to the crop. However, biocontrol agents differ from conventional chemical control in the sense that they are not applied to completely eradicate the pest, but rather to suppress them to a point at which they will not affect the crop yield (Hynes and Boyetchko, 2006). Biocontrol agents have become an important alternative to chemical pesticides for control of crop pests including diseases, weeds, and insect pests. Environmental authorities as

well as the general public have expressed concerns over the use of some chemical pesticides that may be harmful to the environment and human health (Johansen and Olsson, 2005). The pesticide regulatory departments of both European and North American governments have plans to deregister many chemical pesticides within the next few years, leading to a renewed interest in biocontrol as an alternative to these chemical methods (Hynes and Boyetchko, 2006).

Weeds are a significant contributor to yield loss in economically important crops and chemical pesticides have long been used to suppress weed growth and enhance crop yield (Boyetchko *et al.,* 2002). Environmental concerns include soil and water contamination with chemical residues, habitat loss, and negative impacts on non-target organisms, herbicide resistant weeds, spray drift, and impacts on biodiversity (Mark *et al.,* 2006). Consumers are also concerned with the potential detrimental effects of chemical residues in food products (Mark *et al.,* 2006).

There are several characteristics that one should look for when developing a biocontrol agent. These include ease of application, genetic stability, cost-effective mass-production, long shelf life, fast acting, and predictable field performance. A good biocontrol agent should also effectively inhibit pathogens, have a narrow host range, and it should persist in the environment long enough (ie. entire crop season) to provide effective control of pests (Boyetchko and Peng, 2004).

The majority of microorganisms used as biocontrol agents of weed pests (bioherbicides) are fungal pathogens, but there are an increasing number of bacterial pathogens being developed (Boyetchko *et al.,* 2002). Bioherbicides are applied to fields in a similar way as chemical herbicides, with the microorganism and its metabolites being

the active ingredient. While bioherbicides may not completely replace chemical herbicides they provide an additional tool in the weed management strategy. Integrated weed management is based on the notion that complete eradication of weeds is not the best method, but reducing weed levels below economically significant thresholds will result in a more environmentally sustainable crop production system (Boyetchko *et al.,* 2002).

2.2 Examples of bioherbicides

As of 2005, nine bioherbicides have been either registered or given permission for use around the world and an additional two were in the registration process. The first two bioherbicides registered in the U.S. were Collego® and Devine®. *Colletotrichum gloeosporioides* f.sp. *aeschynomene* was the active ingredient in Collego® and was used for the control of northern jointvetch (*Aeschynomene virginica*) in rice and soybean fields. However, Collego® was deregistered in 2003 because of its narrow host specificity, which limited its commercial success (Boyetchko, 2005). Devine® contains *Phytophthora palmivora* for the control of stranglervine (*Morrenia odorata*). Devine® is able to persist in the soil long-term, which is both beneficial and detrimental because it may cause problems in crop rotations if a new crop is introduced which is a target of *P. palmivora.* The first bioherbicide to be registered in Canada was BioMal®; however, its commercial development, like Collego®, was terminated. This was due to difficulties in cost-effective mass-production (Boyetchko, 2005).

Other bioherbicides that have been successfully commercialized include Camperico® and Stumpout®, which contain the bacterium *Xanthomonas campestris* pv.

poae and the fungus *Cylindrobasidium leave,* respectively. Camperico® is used to control annual bluegrass (*Poa annua*) in golf courses and Stumpout® to control wattle (*Acacia mearnssi* and *Acacia pycnantha*) in South Africa. Although highly host specific products such as Collego® were unable to achieve commercial success, Camperico® has been commercially successful because it has a high value market in the golf course industry (Boyetchko, 2005).

Various strains of *Chondrostereum purpureum* have been formulated into biocontrol products. For example, Biochon™ is commercially available in the Netherlands to prevent the sprouting of birch, aspen, and willow in plantations. Chontrol™ and is registered in Canada and the U.S. for the inhibition of resprouting from cut stumps of red and sitka alders in railways, ski resorts, and tree farms. A third strain of *C. purpureum* has been registered in Canada for deciduous tree sprouting control as the bioherbicide MycoTech® Paste (Boyetchko, 2005).

2.3 P*seudomonas fluorescens*- a potential biopesticidal species

Bacteria belonging to the genus *Pseudomonas* are characterized as Gram-negative, rod-shaped with polar-flagella and belong to the gamma subclass of Proteobacteria. Pseudomonads are ubiquitous in soil, water, and plant surfaces. Many live in commensal relationships with plants. They benefit the plant by suppressing pests, enhancing availability of nutrients, or degrading environmental pollutants. Many pseudomonads produce a variety of secondary metabolites capable of suppressing the growth of certain plant pathogens and consequently indirectly promoting plant growth (Paulsen *et al.,* 2005).

For instance, *Pseudomonas fluorescens* Pf-5 produces the secondary metabolites pyrrolnitrin, pyoluteorin, and 2,4-diacylphloroglucinol, which are toxic to oomycetes, for example, *Pythium*. It also produces other compounds such as hydrogen cyanide and the siderophores pyochelin and pyoverdine which suppress pathogens through iron competition. Pyoverdine is the class of siderophore which contain a chromophore responsible for the UV fluorescence characteristic of fluorescent pseudomonads. Also, lipopeptides produced by some *Pseudomonas* spp. have surfactant and antibiotic properties that also contribute to protection from pathogens (Paulsen *et al.,* 2005).

Pseudomonas spp. have been developed as commercial biocontrol products. They are of particular interest because of their ability to colonize the rhizosphere in high numbers. Many fungal pathogens present in the rhizosphere can attack plant roots and cause significant economic losses. Pesticides are rarely effective in preventing these root diseases, therefore root-colonizing bacteria such as pseudomonads can inhibit pathogens through production of secondary metabolites (Baehler *et al.,* 2005). They compete well with soil-borne pathogens and produce secondary metabolites with powerful antifungal properties. Some examples are the products BioJet Spot-Less manufactured by EcoSoil Systems Inc. and BlightBan manufactured by Nufarm Inc., which contain *Pseudomonas aureofaciens* and *Pseudomonas fluorescens*, respectively. BioJet Spot-Less targets the pathogen *Sclerotinia homeocarpa,* a pathogen of turf. BlightBan targets the almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry and tomato pathogen *Erwinia amylovora* (Mark *et al.,* 2006).

Pseudomonas fluorescens CHAO produces three antifungal metabolites; 2,4-diacetyl-phloroglucinol (DAPG), pyoluteorin (PLT), and pyrrolnitrin (PRN), which all possess broad-spectrum anti-fungal activity. PRN is effective at controlling several pathogens such as *Rhizoctonia solani* and *Sclerotinia homeocarpa*. DAPG is able to suppress several soil-borne diseases such as black root rot of tabacco caused by the pathogen *Thielaviopsis basicola* and take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici.* PLT is able to control root diseases caused by various members of the genus *Pythium* (Baehler *et al.,* 2006).

Another strain, *Pseudomonas fluorescens* 2-79 is a biocontrol agent of take-all, a root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* which causes significant losses and occurs world-wide, yet methods to control this disease are limited. This strain produces the secondary metabolite phenazine-1-carboxylic acid (PCA) which suppresses the growth of the pathogen. Pseudomonads are able to colonize the roots limiting the infection and spread of this pathogen. *Pseudomonas chlororaphis* 30-84 also produces PCA to control take-all, along with two other phenazines, 2-hydroxy-phenazine-1-carboxylic acid and 2-hydroxyphenazine (Slininger *et al.,* 1996).

In addition to the ability to suppress the activity of microbial pathogens, *P. fluorescens* can serve as a biocontrol agent of weed plants. *Pseudomonas fluorescens,* as well as some other rhizobacteria are capable of suppressing the growth of some weed plants, which is beneficial to crop plants because by stunting the development of the weed population there is less competition for light, water, and nutrients (Kremer *et al.,* 1990; Kremer and Kennedy, 1996). The goal with the use of rhizobacteria as biocontrol

agents is to suppress weed growth rather than eradicate them completely (Daigle *et al.,* 2002).

It has been reported that specific rhizobacteria which suppress weed growth are ubiquitous and probably found in all plant rhizospheres (Kremer *et al.,* 1990). Other examples of rhizosphere bacteria with bioherbicidal activity are *Enterobacter*, *Arthrobacter* and *Pseudomonas cichorii* (Boyetchko *et al.,* 2002). This group of bacteria probably cause damage to the weed plant through the production of phytotoxins that are taken up through the plant roots (Kremer *et al*., 1990). It has been reported that over 90% of fluorescent bacteria found in citrus root systems possess siderophore activity, which are likely involved in the suppression of weed growth (Kremer *et al.,* 1990). In laboratory studies, over 100 bacterial isolates have been found to suppress the growth of grass weed roots by 80% (Daigle *et al.,* 2002). According to Kremer *et al.* (1990), rhizobacteria will be successful in suppressing weed growth if they have a high colonizing ability, produce specific phytotoxin(s) that suppress growth of the host weed, are not suppressed by siderophores or antibiotics produced by competing microorganisms, and have the ability to synthesize siderophores.

Pseudomonas fluorescens BRG100 is a pathogen of wild oat (*Avena fatua*) and green foxtail (*Setaria viridis*). Wild oat is one of the most important grassy weeds in the Canadian prairies. Total annual losses caused by wild oats the prairies may be as high as $500 million (Sharma and Vanden Born, 1978). Green foxtail is another annual grassy weed, which is a weed of corn, soybean, cereals, canola, sugar beet, and pastures (Daigle *et al.,* 2002). The suppression of weeds by *P. fluorescens* BRG100 has been attributed to secondary metabolites and phytotoxins. As mentioned previously, *Pseudomonas* spp.

possess the ability to produce a variety of metabolites. This includes phytotoxins that cause symptoms such as root discolouration and reduced root length and also affect lipid synthesis and membrane integrity (Boyetchko *et al.,* 2002).

2.4 Formulations for biocontrol agents

Bacteria are usually mass-produced using liquid fermentation systems, but can also be produced through semisolid or solid-state fermentation (Boyetchko *et al.,* 1999). Important conditions that must be considered are oxygen transfer, incubation temperature, nutrient requirement, and agitation to ensure a large, stable, and efficacious bacterial population. The nutrients added to the medium should be inexpensive, readily available, and conducive to a high biomass and proper secondary metabolite production (Hynes and Boyetchko, 2006).

A major obstacle to the widespread use of microorganisms as biocontrol agents is the lack of effective and economical formulations. Among the many attributes of a commercial biocontrol agent, an adequate shelf life is very important in order for it to be accepted. The shelf life of a biocontrol agent refers to the period of time that the microbial agent remains viable and infective (Elzein *et al.,* 2004). The longer the shelf life, the greater the chance the product will be registered and commercialized (Shabana *et al.,* 2003).

When formulating biocontrol agents, there are a number of factors that must be addressed: the ease of production and application, as well as sufficient product stability and shelf life during transportation and storage over long periods. The appropriate formulation can improve the viability and stability of the active microbial agent.

Furthermore, it is important to formulate biocontrol agents into a form compatible with existing farm equipment and management practices in order to ensure acceptance of the new product (Boyetchko *et al.,* 1999).

The ingredients added to the formulation must ensure stability during processing and storage, as well as protection from harmful environmental conditions. The formulation of a biocontrol agent is composed of the active ingredient, carriers which are inert materials used to deliver the active ingredient to the target, and lastly the adjuvants, which are compounds that promote and sustain the active ingredient by protecting from harmful conditions such as UV radiation and desiccation (Hynes and Boyetchko, 2006).

The active microbial agent in a biocontrol product can be a bacterial cells, fungal mycelia and spores, or virus particles. The target pest may be a fungal or a bacterial pathogen, a weed plant, an insect or a nematode. The final product can be in a solid, liquid, slurry, powder or granular formulation. Liquid formulations may be oil, aqueous, or polymer based. Aqueous formulations usually require the addition of ingredients such as stabilizers, surfactants, and additional nutrients. Oil-aqueous based formulations are blended with a mineral or vegetable oil based carrier and an emulsifier. Oil based formulations reduce evaporation of the product, but too much oil is phytotoxic to crop plants. Formulations may also be dry or wettable powders, or granular. These formulations are created by spray drying, freeze drying, or air drying in a fluidized bed. This type of formulation requires addition of binders and wetting agents. The formulation should be protective of the microorganism, ensuring high viability and a long shelf life (Boyetchko *et al.,* 1999).

Biocontrol agents can be applied in a number of different ways. They can be seed, soil, or foliar applied. They can be pre-coated onto the seed, or as with dusts, powders, or granular formulations, incorporated into the soil. Wettable powders and liquid formulations can be applied in the furrow. Control agents can be delivered via foliar applied biocontrol agents are prepared in a liquid formulation and applied at high volume to ultra low volume (Boyetchko *et al.,* 1999).

As with bacterial biocontrol agents, fungal biocontrol agents can be formulated into granules, pellets, or wettable powders. Fungal pathogens that infect the roots of weeds are best suited for granular or solid formulations. Granular formulations are advantageous because they can buffer the microbial agent from environmental extremes; provide nutrients for survival and growth following application, and enable longer persistence. They also allow controlled release of the microorganism from the formulation which means it can replicate and grow to the site of action when suitable environmental conditions are present. This conserves the energy resources of the organism until the optimal time to infect (Shabana *et al*., 2003).

Viruses can also be used as biocontrol agents for insect pests. Most viruses are formulated into wettable powders. Viruses are advantageous because they are highly specific and do not affect beneficial insects. They also are able to persist in the environment for considerable periods of time. However, viruses are slow acting, can be unstable under UV light, and are difficult to mass-produce (Boyetchko *et al.,* 1999).

2.4.1 Powder and granular formulations

There are several advantages of using powder or granular-based formulations as opposed to liquid formulations. Liquid formulations are best for applying post-emergence

biocontrol agents. Solid formulations are best for pre-emergent biocontrol agents, which attack below the soil surface. Some of the advantages of solid formulations are as follows: extended shelf life, a buffering capacity against extremes in temperature and moisture, they are less likely to be washed away by rain or water or to be blown away by wind, and ingredients in the formulation can be used as a source of nutrients for the active ingredient (Daigle *et al.,* 1997).

2.4.2 Liquid formulations

Foliar applied fungal biocontrol agents are very sensitive to environmental conditions such as temperature and moisture and usually are dependant on a dew period for germination and infection. The addition of certain ingredients such as corn oil increases activity and reduces dew period requirements. Another important ingredient is a surfactant. Vegetable oil and surfactant help to wet plants by reducing surface tension and improving spore dispersal. Examples of surfactants are Tween 20 (polysorbate 20) and sorbitol. Foliar fungal biocontrol agents can be effective when prepared in water-in-oil emulsions, which provide a good environment for germination and infection (Boyetchko *et al.,* 1999).

2.5 The pesta formulation

The pesta granular formulation is an extruded product composed of grain flour and the microbial agent. Pesta technology is adaptable to many different microorganisms and ingredients (Daigle *et al.,* 1997). The formulation is usually a wheat gluten matrix derived from inexpensive wheat flour, kaolin, nutritional adjuvants, and water, which

house the microbial agent (Daigle *et al*., 2002). Once these ingredients are combined, the dough is extruded through a small pasta-making machine into thin sheets. These sheets are air-dried and sieved to a specific size. The granules are usually between 0.6 mm and 1.4 mm in diameter (Connick *et al.,* 1991). High-speed extrusion machinery used to make foods such as spaghetti can be used to produce pesta in large-scale operations (Daigle *et al.,* 1997, Boyetchko *et al*., 2002). In addition to the ability to be produce pesta on a large scale, there are several other advantages to the pesta formulation. It is non-toxic, cost effective, convenient to store, simple to use, and can be applied with agricultural machinery (Elzein *et al.,* 2004).

The pesta formulation was developed by Connick *et al.* (1991) and has been used to formulate many different antagonistic fungi such as *Alternaria cassiae, Alternaria crassa, Colletotrichum truncatum*, and *Fusarium lateritium* used for the control of the weeds sicklepod, jimsonweed, hemp sesbania, and velvetleaf, respectively (Elzein *et al.,* 2004). The most important factors affecting shelf life are temperature and moisture content or water activity. The water activity is a useful measure of the free or unbound water available to be used by the microorganism. It is equal to the equilibrium relative humidity in the sealed container divided by 100. It is critical to the shelf life of pesta that the water activity is between 0.2 and 0.4. This value is below the critical A_w 0.6-0.7 range in which contaminating microorganisms cannot grow (Hahn-Hagerdal, 1986)

In a study by Elzein *et al.* (2004) pesta granules were prepared from semolina, which is a coarse durum wheat flour, and kaolin. The liquid fungal inoculum was added to these ingredients and mixed by hand to form cohesive dough. This is a distinct advantage that the liquid fermentation culture can be used to supply water and fungal

inoculum simultaneously, reducing time and cost. The dough was kneaded, flattened, and folded several times. It was passed through a pasta maker, folded and then passed through the pasta maker several more times. The dough sheet was laid on a polyester window screen and allowed to air-dry. Once dried, the sheets were ground with a hand-operated grinder and sieved to a uniform size of 0.6- 1.4 mm diameter (Elzein *et al.,* 2004). Another advantage is that the inoculum entrapped in the pesta granules produce new propagules through solid-state fermentation when the temperature and moisture levels are adequate, unlike simple conidial sprays of a fixed concentration (Connick *et al.,* 1991).

The high concentration of inoculum provided by the pesta formulation allows the entrapped microbial agent to proliferate and infect the weed more readily than the naturally occurring competing microorganisms present in soil in fewer numbers. The production of pesta is amenable to the addition of many adjuvants, which may alter certain properties of the formulation such as density, hardness, and moisture level, as well as to provide additional nutrients (Shabana *et al*., 2003).

Processing strategies that reduce loss of viability caused by severe grinding, low water content, and drying are needed. The twinscrew-extrusion of dough used in the food industry to produce spaghetti can be used to control the size and shape of the final formulation making the production of small granules possible without destructive grinding processes, which can damage the fungal propagules, thus reducing viability. (Shabana *et al*., 2003).

Pesta granules have been prepared using twin-screw extrusion followed by fluid bed air-drying. In a study by Daigle *et al.* (1997) the weed hemp sesbania pathogen *Colletotrichum truncatum* was formulated into pesta granules in this manner. Fluid bed

air-drying was used because it was fast and homogenous. It reduced the number of lumps and the chance of uneven heating (Daigle *et al.,* 1997).

The pesta formulation was found to be a suitable formulation for the use of *Fusarium oxysporum* f.sp. *orthoceras* (FOO) as a bioherbicide of sunflower broomrape (*Orobanche cumana*). Important parameters of processing were the water activity and the temperature. Low water activity of the final product and low storage temperature resulted in extended shelf life. While the water activity was relatively easy to control in small laboratory samples, the drying of large amounts of FOO-containing pesta for use as a commercial bioherbicide to low water activities without significant loss of viability is a great challenge to the industry (Shabana *et al.,* 2003).

In another study, *Fusarium oxysporum* was formulated into pesta granules (Elzein *et al.,* 2004). The researchers found that by manipulating the water activity and sucrose content, the viability and stability of the product could be improved. *Fusarium oxysporum* retained 50% viability after one year of storage in a refrigerator. When stored at room temperature, a low water activity of 0.12-0.34 was found to be optimal for retention of viability. Storage at low temperatures was easier and more economically effective than maintaining low water activity over a long period of time (Elzein *et al.,* 2004).

Colletotrichum truncatum is a fungal pathogen used to control hemp sesbania (*Sesbania exaltata*), a weed of crops such as cotton and rice. In a study by Connick *et al.* (1996) the longest shelf life of *C. truncatum* formulated into pesta granules was determined to be optimal in samples in the water activity range of 0-0.33. At this level the

water is bound in the matrix and is unavailable to the fungus. Samples stored at water activities of 0, 0.12, and 0.33 had 100% viability after 24 weeks (Connick *et al.,* 1996).

Pseudomonas fluorescens BRG100 has been formulated into a pesta granular formulation using a single screw extruder followed by fluidized bed drying. The best formulation in this case was oat flour with 20% w/w maltose. Pesta provides an extended shelf life of up to 32 weeks. A field trial in 2000 was conducted to examine the biological control efficacy of BRG100 formulated into pesta granules. The concentration of bacterial cells in the pesta granules was 7.2 x 10^7 cfu/g and the amount applied was 50 g/row. This concentration resulted in 74% reduction of green foxtail emergence at the AAFC Saskatoon research farm. In a similar trial at the AAFC Scott, SK research farm the suppression was as much as 83% when applying 35 g/row of pesta granules with the same concentration (Daigle *et al*., 2002).

2.6 Green fluorescent protein as a molecular marker

The use of molecular markers for biocontrol agents has enabled researchers to track microorganisms after release into the environment (Vurro and Gressel, 2006). Genetically engineered microorganisms are being developed for use in agricultural pest control. Molecular detection methods including gene probing, DNA hybridization, polymerase chain reaction (PCR) and reporter genes are being used in addition to traditional detection methods. Molecular markers allow for the specific strain to be differentiated from others.

Green fluorescent protein (gfp) is a useful marker for microorganisms, allowing new research that will increase our understanding of microorganisms interaction with the environment (Errampalli *et al.,* 1999). In 1994, a new marker system, the green

fluorescent protein was developed. Research on gfp has been carried out for over 35 years and in the past decade(s) gfp has attracted considerable attention as a marker/reporter system (Errampalli *et al.,* 1999**)**. Green fluorescent protein is a small protein of 27 kDa isolated from the bioluminescent jellyfish *Aequorea victoria*. The property of interest is that gfp converts the blue chemiluminescence of the Ca^{2+}-sensitive photoprotein, aequorin, into green light. It absorbs blue light at 395 nm and emits green light at 510 nm. UV or short-wave blue light excites it (Errampalli *et al.,* 1999).

There are many characteristics that make green fluorescent protein more attractive than other genetic markers such as the *lux*AB luminescence marker, which requires aldehyde substrates to be visualized. Green fluorescent protein is stable in the presence of many denaturants and proteases, at pH values of 6-12, and persists at temperatures as high as 65°C (Errampalli *et al.,* 1999).

The chromophore is made up of three contiguous amino acids near the amino terminal end of the protein. A chromophore is the part of the molecule responsible for its colour. Green fluorescent protein has been used in many studies to visualize the growth, colonization, and persistence of soil bacteria. For example, gfp was used to visualize the events in the symbiosis between *Rhizobium* bacteria and alfalfa (Gage *et al.,* 1996). *Rhizobium meliloti* constitutively expressing the *gfp* gene was visualized as it grew in the rhizosphere, colonized the root surface and entered infection threads (Gage *et al.,* 1996). Green fluorescent protein is useful because cells can be visualized non-destructively and without adding exogenous substrates (Bloemberg *et al.,* 1997).

There are many advantages in using gfp as a marker such as: no requirement for an outside energy source, visualization through confocal scanning laser microscopy has no

requirement for fixing and staining as would be needed with regular light and electron microscopy, and no processing of the cells is needed. Green fluorescent protein has been stably maintained in *Pseudomonas* spp. in past studies (Bloemberg *et al*., 1997).

Direct microscopy of bacteria marked with gfp is simple with the use of an epifluorescent microscope. Green fluorescence can be visualized in colonies on agar media under a hand-held long-wave (365 nm) UV lamp. This is a simple approach that can be used to enumerate *gfp*-labelled microorganisms from environmental samples. Confocal laser scanning microscopy can be used to visualize *gfp*-marked microorganisms with high resolution, and with 3D image analysis. This allows the visualization of three-dimensional distribution of bacteria in complex communities (Errampalli *et al.,* 1999).

2.7 Root colonization by *Pseudomonas fluorescens*

Inconsistent root colonization by biocontrol agents may limit efficacy in field conditions. By identifying the traits that enable successful root colonization, a more efficient biocontrol product can be made. The identification of these traits is dependent on a good understanding of the localization, organization, activity and viability in the rhizosphere along the root (Gamalero *et al.,* 2005).

There are many biotic and abiotic factors that contribute to the colonization process by microorganisms in the rhizosphere. These factors range from the type and amount of root exudates produced by the plant and the ability of the micoorganisms to utilize these sources to the motility of the microorganisms that can affect the way in which the roots are colonized (Rincon *et al.*, 2005). Therefore examination of the spatial distribution and colonization patterns can provide vital information. For example, if a bacterium is found

to preferentially colonize the roots at a particular site there may be a concentration of root exudates being released from the plant in that area (Rincon *et al.*, 2005). The type and amount of root exudates are influenced by plant age and zone of release and this in turn will suppress or stimulate the growth of certain bacteria. This results in changes in the bacterial species composition, as well as the proportion of viable and dead cells, which will vary over time and over the length of the root (Gamalero *et al.,* 2005).

The age of the root cells also play a major role in the colonization patterns. It is therefore of interest to understand the way in which a root grows. Early root growth is a function of the apical meristem, which is located close to the tip of the root. The meristem cells continuously divide, producing both meristem and root cap cells, which protect the meristem. The root cap provides mechanical protection to the meristem. As its cells are worn away, new cells are generated within the meristem. The root cap also produces mucigel, sticky mucilage that coats the new cells. The meristem also produces undifferentiated cells that will undergo elongation, pushing the root tip forward in the soil. These cells will eventually differentiate and mature into specialized root cells (Taiz and Zeiger, 2002). Because of such a high turnover rate of cells of the root tip and meristem, it is common to find an increase in the amount of microorganisms colonizing this region.

In past studies, various strains of *P. fluorescens* have been transformed with gfp and their colonization pattern in the rhizosphere of many plant species has been examined (Gamalero *et al.,* 2005). Tombolini *et al.* (1997) reported that *gfp*-tagged pseudomonads could still be observed by flow cytometry in soil even in nutrient deprived situations,

suggesting gfp is a very useful marker in the rhizosphere even in field conditions (Tombolini *et al.,* 1997).

Rhizosphere microorganisms are usually found in aggregates, due to different amounts and types of root exudates at different areas on the root zone (Dandurand *et al*., 1997). The authors found that *P. fluorescens* 2-79RN$_{10}$ and *P. fluorescens* 2-79-B46 formed aggregates while colonizing the roots of pea plants. The aggregates were found consistently along the length of the root although the bacterial density decreased towards the root tip. There was an absence of bacteria at the root tip and aggregates were found most commonly along the length of cell walls (Dandurand *et al*., 1997).

Areas of the root with the highest amount of exudates have been reported to be the region of elongation, the apical meristem, the region of developing roots, and the root tip. However, the results found by Dandurand *et al*. (1997) did not correspond to these reported exudate hot spots. One explanation for the large numbers of aggregates along epidermal cell walls is that the surface irregularities produce niches where the bacteria are protected from the mechanical shear that normally occurs on smooth surfaces (ie. by flowing water, during root growth through the soil, the movement of other organisms, such as nematodes). The results suggest that there are a variety of factors affecting bacterial aggregation on the root such as surface topography, the interactions among the pseudomonads and other microorganisms, as well as nutrient availability via root exudates.

Another area of the plant that provides protection from mechanical shearing is the seed. A study on wheat plant parts revealed the seed is the preferential area of colonization by *P. fluorescens* SBW25 (Unge and Jansson, 2000). The localization of

bacterial cells was determined using *gfp*-marked bacterial cells and a variety of microscopic techniques. *Pseudomonas fluorescens* SBW25 was shown to aggregate on the seed surface preferentially in between the scutellum and coleoptile through analysis using fluorescence stereomicroscopy. In addition, confocal imaging demonstrated a concentration of bacterial aggregates on the rough surface of the ventral side of the seed (Unge and Jansson, 2001).

There are a few different reasons as to why the bacteria prefer the seed. Cracks and grooves on the seed surface provide protection from mechanical forces. Also, at the beginning of seed germination cytoplasmic solutes are leaked via broken plant cells. This provides a large amount of nutrients for the bacteria. Although Unge and Jansson (2001) found that the seed was the preferred colonization site, leaves and roots were also colonized over a 65-day period. Bacterial cells were found mostly on the upper region of the main root. Numbers of cells on the root as a whole decreased over time (Unge and Jansson, 2001).

In a root colonization study by Lugtenberg *et al.* (1999) it was revealed that *P. fluorescens* WCS365 preferred the seed and the region just below the seed on a wide variety of plants. The bacterial concentration decreased down the length of the root with a 100-fold decrease in bacterial cells at the root tip than the seed. Bacteria were also found consistently in microcolonies (Lugtenberg and Dekkers, 1999).

The pattern of root colonization can also be affected by time. Two similar studies on colonization of the roots of tomato plants had very different results. In the first study, g*fp*-tagged *P. fluoresens* 92rkG5 was analyzed at 3, 5, and 7-day intervals on three morphological root zones of tomato plants: the root hair, the collar, and the root tip.

Through the use of confocal laser scanning microscopy and dilution plating, Gamalero *et al*. (2005) found that the three root zones demonstrated different distribution and viability of bacterial cells, but there was no change in the distribution over time. They found root tips to be free of bacteria and with increasing distance from the apex, the number of bacterial colonies steadily increased. Viability was lowest in old zones of the root and highest in the elongation zone (Gamalero *et al.,* 2005).

In the second study the colonization of tomato roots by *P. fluoresens* A6R1 was investigated. Bacteria were found to be present but in changing patterns at 3, 5, and 7-day intervals. In 3-day old plants, bacteria were spread out evenly, while in 5-day old roots, bacteria were present mostly in pairs, indicating that the bacteria had recently divided. On 7-day old roots bacteria were present in strings following the longitudinal epidermal cell walls. Bacterial cells were also found in clusters on root hairs (Gamalero *et al.,* 2004).

Other interesting information on the effect of time on colonization patterns has been discovered. Van Bruggen *et al.* (2007) found a distinct colonization pattern of *P. fluorescens* 32 along the roots of wheat plants. Populations of bacteria were present in moving waves along the root, with distances of about 30 cm between waves. They hypothesized these spatial waves were the result of temporal waves in the release of nutrients by the root tip. A release of nutrients by the root tip would cause an exponential increase in bacterial growth behind the root tip, which would then be followed by a period of death due to nutrient limitation. The spatio-temporal distribution pattern of this biological control bacterium as with others could be very important to their effectiveness. It is best to select a biological control agent that will have the highest population at the area and time of infection by the pathogen (van Bruggen *et al.,* 2007).

The colonization pattern of *P. fluorescens* CS85 on cotton roots had also been examined. The strain colonized all surfaces of the plant root zones: including root hairs and lateral roots, however the distribution along the root was not uniform. The number of bacteria increased in the first two weeks to a cell density of 10^8 cfu/cm in the upper segment of the root and 10^7 CFU/cm in the lower segment. These values remained consistent until 35 days then began to decrease (Wang *et al*., 2004). This is consistent with other plant growth-promoting bacteria in the rhizospheres of corn, tomato, broad beans, barley, and canola (Ma *et al.,* 2001).

2.8 Environmental factors that affect biocontrol agents

The ability of a soil-applied biocontrol agent to control its target pest depends on the survival of the microbial inocula in the soil. A microbial agent may be highly effective under laboratory conditions but be unpredictable in the field. This is because different environmental factors, such as soil temperature, soil moisture, as well as soil type, can all effect the establishment and survival of the microbial inocula in soil. By minimizing the decline of viable cells in the biocontrol product following application, the efficacy of the product can be greatly improved, but this is only possible through understanding the reasons for the decline (O'Callaghan *et al*. 2001). There are a number of environmental factors that affect both the ability of the microbial agent to colonize the roots of target plants as well as its survival in the rhizosphere. These include temperature, moisture content, pH, soil composition, and the activities of other microorganisms (Cools, *et al*., 2001).

Seong e*t al*. (1991) investigated the growth and survival of *P. fluorescens* ANP15 under different temperatures in sandy loam soil. Maximum growth of *P. fluorescens* ANP15 was observed at 18ºC. There was only a slight decrease in the $\log_{10}$ cfu/g over a 50-day period at 4ºC and survival was even better at –18ºC. The maximum growth of *P. fluorescens* 7NSK2 was 28ºC and there was only a slight decrease in the $\log_{10}$ cfu/g over a 50-day period at 4ºC. However, survival at –18ºC was greatly reduced (Seong e*t al*., 1991). Soil temperature also affects both growth and competition of other microorganisms, which in turn affects the growth and survival of the microbial agent (Cools *et al*., 2001).

In addition to soil temperature, soil moisture has been found to affect root colonization ability and survival of *P. fluorescens*. Normander *et al.* (1999) found that the water content of soil had a dramatic impact on the persistence of *P. fluorescens* DR54-BN14 in the barley rhizosphere. In dry soil [<10% water holding capacity (WHC)] the total number of bacterial cells in the rhizosphere remained constant over time, but in wet soil (160% WHC) the total number of bacterial cells was reduced drastically (Normander *et al.,* 1999).

O'Callaghan *et al*. (2001) examined the survival of *P. fluorescens* CHAO-Rif in soil at three soil moistures: 30%, 23%, and 13%, and at 3 temperatures: 10, 15, and 20ºC. In this study *P. fluorescens* CHAO-Rif was significantly affected by both temperature and soil moisture. The survival after 110 days was greatest at 23% moisture and lowest at 13% moisture. The population decline was greatest at 20ºC, becoming undetectable after 54 days at all three soil moistures. The survival was best at 10ºC, regardless of soil moisture content (O'Callaghan *et al*. 2001).

Soil type and texture are also important environmental factors affecting biocontrol agents. A soil's texture influences soil water content and provides microhabitats, formed from organic matter and soil aggregates (Cools *et al*., 2001). Wessendorf and Lingens (1989) concluded that fine textured soils yield higher microbial counts than coarse textured soils, especially at low moisture levels. Soils rich in clay allow more microhabitats to be formed providing enough water and nutrients, as well as protection from predatory microorganisms. The amount of organic matter present in soils is also important to survival. Organic matter helps with water retention, formation of aggregates, and the formation of microhabitats (Wessendorf and Lingens, 1989).

Another important environmental factor is soil pH. Soil pH can affect the growth and survival of microorganisms. It is well known that bacteria do not grow well at low soil pH (<5). Van Bruggen *et al*. (2007) found that populations of *P. fluorescens* 32 were higher in soils with higher pH. They incubated the strain in two sandy soils one from a conventional farm and the other from an organic farm with similar characteristics except for a significantly different pH of 6.8 and 5.3, respectively. The textures and clay content of the soils were very similar (Van Bruggen *et al.*, 2007).

Cools *et al*. (2001) examined the survival of *E. coli* and an *Entercococcus s*p. in 3 soils of different texture: sandy, loamy, and loamy sand; at 3 incubation temperatures: 5, 15, and 25ºC; and 3 soil moisture contents: 60, 80, and 100% of field capacity. For both species lower temperatures enhanced survival. Lower temperatures may have favoured survival of *E. coli* and *Entercococcus s*p. due to a reduction in the activity and competition for nutrients of other microorganisms in the soil. Sandy soil was best for *E. coli* survival, while loamy soil was best for *Entercococcus s*p. survival. For both bacteria,

higher moisture favoured survival. This is most likely because a reduction in water content could cause desiccation and drought stress and may lower the bacterial population (Cools *et al.,* 2001).

However, root colonization and survival of microbial biocontrol agents are sometimes independent of some environmental factors. In a study by Wang *et al.* (2004), the colonization of cotton roots and survival over time of *P. fluorescens* CS85 was found to be independent of soil type and soil pH. Growth and survival in a sandy loam (pH 8.5) was very similar to a silt loam soil (pH 5.2) (Wang *et al.,* 2004).

Formulations developed for biocontrol agents help overcome the environmental obstacles experienced after introduction into the soil. This can be in the form of carriers, selective food sources, or buffers that can provide a temporary protection to the microbial inocula for a period until conditions permit the biocontrol agent to establish in the soil. The microbial inoculum is therefore able to adjust physiologically to the new surrounding soil conditions. O'Callaghan *et al*. (2001) reported that the survival of *Serratia entomophila* 626 was greatly improved after formulation into granules and placement into soil with different moistures and temperatures, as compared to a similarly treated liquid suspension of *S. entomophila* 626. Thus, formulation is one method of overcoming environmental stresses to biocontrol agents (O'Callaghan *et al*. 2001).

3 Materials and Methods

3.1 Strains and culture conditions

Pseudomonas fluorescens BRG100 (hereafter referred to as BRG100) was used as the reference and parental strain for all experimental trials. BRG100 was isolated from an infected green foxtail seed during a routine germination assay at Agriculture and Agri-food Canada (AAFC) on July 7, 1995. All *P. fluorescens* strains were cultivated by streaking onto nutrient agar (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at room temperature for 2-4 days. *Escherichia coli* pAG408 was cultivated by streaking on Luria-Bertani (LB) agar plates (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at room temperature for 2-4 days.

3.2 Culture storage

BRG100 and all 16 *gfp*+ isolates (described in section **3.3**) were stored in cryovials containing sterile 20% glycerol solution in the ultra low temperature (-86°C) freezer (Forma-Scientific, Inc. Marietta, OH, USA). The 16 *gfp*+ isolates were frozen according to the protocol used by AAFC, as described below.

The freezing protocol used is as follows. Single BRG100*gfp*+ colonies were picked from kanamycin-containing LB agar plates (Sigma-Aldrich, St. Louis, MO, USA) and inoculated into centrifuge tubes containing 20 mL of nutrient broth (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) with 50 mg/L kanamycin. The tubes were vortexed using the S/P Vortex Mixer, speed 10 (Baxter Scientific Products, Deerfield, IL,

USA) and placed on the rotary shaker at room temperature at 150 rpm for 48 hours. The tubes were removed and centrifuged for 10 minutes at 4100 rpm. The supernatant was discarded and 1 mL of phosphate buffer (Fisher Biotech, Fair Lawn, NJ, USA) (see **Appendix F**) was added to the pellet in order to wash the bacteria and improve viability in the freezer. The tubes were vortexed and centrifuged for 5 minutes at 4100 rpm. The supernatant was discarded and 3 mL of 20% v/v glycerol was added. The tubes were then vortexed.

Five hundred µL of the culture broth was added to each of 5 cryovials and placed in the refrigerator at 4ºC for 15 minutes. The tubes were then placed in the freezer at –20ºC for 15 minutes and then placed in the ultra low freezer at –86ºC for long-term storage. Quality control tests were conducted by streaking onto *Pseudomonas* agar F (PAF) plates (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) and nutrient agar plates from the remaining bacterial culture in the centrifuge tubes from the previous step in order to ensure purity of the bacterial culture. The plates were incubated at 15ºC for 5 days and examined.

Frozen cultures were confirmed to be pure BRG100*gfp*+ colonies by inspection with a Blak-Ray UVL-21 long-wave UV lamp (Ultra-Violet Products, Inc., San Gabriel, CA, USA). The colonies were further confirmed as pure BRG100 through visual inspection under the Wild M28 dissection microscope, magnification 1.0x, (Leica Microsystems Inc., Bannockburn, IL, USA) for distinct morphological characteristics. Five vials of each isolate were frozen in the ultra low freezer at –86°C. Two of these five vials served as master stock, never to be opened, while the remaining vials were used as working cultures to conduct various experiments.

3.3 Construction of *gfp* mutants

BRG100 was transformed according to the protocol of Goldberg and Ohman (1984) with some minor modifications. The protocol was as follows: *Escherichia coli* S17-λ1 cells that had been previously transformed with the Tn5 mini-transposon suicide plasmid pAG408, which contains the gfp gene, were used to transform BRG100. *Escherichia coli* pAG408 was streaked out on LB agar plates containing 50 mg/L kanamycin, 30 mg/L gentamycin (Sigma-Aldrich, St. Louis, MO, USA), and 50 mg/L ampicillin (Sigma-Aldrich, St. Louis, MO., USA) and incubated at room temperature for 72 hours. BRG100 and a control strain, *P. fluorescens* 840406-E were streaked out on LB agar plates and incubated at room temperature for 72 hours.

Single colonies from each of the three bacterial strains were picked and used to inoculate flasks containing 50 mL of LB broth (Difco™, Becton, Dickinson and Company, Sparks, MD, USA). The flasks were placed on the rotary shaker at 150 rpm for 24 hours at room temperature. One mL of broth from each flask was added to three 1.5 mL centrifuge tubes and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded and 1 mL of 0.9% NaCl was added to the pellet and the tubes were vortexed. The tubes were centrifuged again at 14,000 rpm for 10 minutes.

The supernatant was discarded and 1 mL of NaCl was added. The tubes were vortexed. One mL of the *E. coli* culture was poured into two 50ml tubes, the first containing 1 mL of BRG100 and the other containing 1 mL *P. fluorescens* 840406-E. The tubes were vortexed then membrane filtered using a Nalgene membrane filter device (Nalgene™, Rochester, NY, USA) with a pore size of 0.20 microns.

The filters were placed on LB agar plates for 24 hours, then washed with 1 mL of 0.9% NaCl. The liquid was put into a 1.5 mL centrifuge tube and vortexed for 10 minutes at 14,000 rpm. The supernatant was discarded and 1 mL of NaCl was added. The tubes were vortexed and 100 µL of each of the two combined cultures was added to LB agar plates containing 50 mg/L kanamycin. The plates were incubated at room temperature for 48 hours and examined under UV light for the presence of *gfp* fluorescing colonies. *Gfp*+ colonies were streaked out on PAF to confirm that the transformed bacteria were *P. fluorescens* BRG100 and not *E. coli.*

3.4 Selection of *gfp*+ mutant strain

3.4.1 Comparison of morphology

The selected *gfp*-labelled mutants were streaked out onto nutrient agar plates, along with the wild type. The mutants were compared to the wild type for colour, shape, and overall colony appearance. Only those *gfp*+ isolates that showed a high degree of similarity to the wild-type were selected for use in further experiments.

3.4.2 Comparison of biocontrol efficacy with green foxtail: Growth pouch assay

Each of the 16 *gfp*+ isolates was compared to *P. fluorescens* BRG100, the wild-type, using the growth pouch assay used by AAFC using green foxtail. The protocol used was as follows. A CYG™ Germination Pouch (growth pouch) (Mega International, St. Paul, USA) was suspended within a support box with two clips attached to opposite sides

of the pouch (the pouch top should protrude approximately 15 mm above box). Fifteen seeds were deposited inside the trough of each pouch. Twenty ml sterile water was added to each pouch (dispensed between plastic and paper wick to minimize seed placement disturbance). Seeded pouches were placed in the dark for 2 ½ days (60 ± 4 hours), at room temperature, to initiate seed germination. Ten ml of 10% Hoagland's plant nutrient solution was added to each pouch (see **Appendix A**).

Flasks containing M9 molasses media (see **Appendix B**) were inoculated with each *gfp*+ and the wild-type isolates and placed on the rotary shaker at 14ºC and 150 rpm for the same duration as the seed germination (60 +4 hours). Two ml of the resulting bacteria-culture broth suspension was carefully added over the seedlings. An uninocluated control was also used: 2 ml M9 molasses broth only (no bacteria). Ten ml of 10% Hoagland's plant nutrient solution was added to each pouch. The growth pouches were placed in a light chamber within 1 hour of inoculation. The light cabinet had the following conditions: a 16-hour light period with an incubation temperature of 20°C, followed by an 8 hour dark period at 15°C, with artificial light radiation (8 x 40W fluorescent, Sylvania Design 50, full spectrum) and a relative humidity of 30-50%. The pouches were incubated for 6 days. Plants were monitored daily and watered as required (the trough area should not dry out).

In order to calculate the plant suppressive activity of the microbial strains, 10 plants were removed from each growth pouch by cutting away the plastic. Plants were transferred aseptically to a graduated grid-lined sheet with embryos aligned on the zero line. The plants were measured and the root lengths were recorded.

The plant suppressive activity of each of the 17 bacterial strains (16 *gfp*+ isolates and the wild-type) was determined by comparing the growth of bacteria-treated plants (treatment) to the growth of uninoculated plants (control). The uninoculated control was treated with culture medium only and was considered to have 0% suppression.

The average root measurement for each of the 17 isolates (ten plants per pouch and three replicate pouches per treatment) was calculated. For each treatment, the root measurements were subtracted from the uninoculated control measurement and then divided by the uninoculated control measurement. The dividend was then multiplied by 100 to obtain the 'percent' suppression.

Example: Percent suppression (%) calculation.
[(Untreated control – Treatment)/Untreated control] x 100 = % suppression
e.g. [(100mm – 50mm)/100mm] x 100 = 50% suppression

The ability to suppress growth and cause the characteristic disease symptoms of BRG100 wild-type was investigated. Only the *gfp*+ isolates having an equal ability to suppress the root growth of green foxtail seedlings as the wild-type were used in further experiments.

3.4.2.1 Growth pouch assay- Statistical analysis

The experimental design was a randomized complete block design. There were 18 treatments (16 *gfp*+ isolates, wild type, and untreated control) and three replicates (growth pouches). Each experiment was conducted twice. Statistical analysis was performed with SAS using GLM. A t-test (Fisher's protected LSD test) was used to determine whether means were significantly different at a significance level of $p<0.05$.

3.4.3 Comparison of carbon utilization

BIOLOG™ GN plates were used to determine the ability of the wild-type and the 16 *gfp*+ isolates to utilize various carbon compounds as sole carbon source. The profiles obtained for each of the 16 *gfp*+ isolates were compared to the wild-type and only those most similar isolates were selected for further use. The BIOLOG™ GN plate contains 96 wells. Well A1 is a negative control, whereas wells A2 to H12 each contain a different carbon source (see **Appendix C**).

To evaluate sole carbon source utilization potential of the *gfp*+ isolates and wild-type, the following protocol was used. All isolates were incubated on a shaker in 50 ml centrifuge tubes containing 20 ml of nutrient broth for 24 hours. Tubes were then centrifuged for 10 minutes, the supernatant discarded, and 20 ml of 0.9% NaCl added. The tubes were vortexed and then centrifuged again for 10 minutes, and the supernatant discarded. An appropriate amount of sterile 0.9% NaCl was added to bring the concentration to an optical density of 0.5 A_{600} as determined with the Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, UK). One hundred and fifty µl of each isolate was aseptically added to each well of the 96-well BIOLOG™ GN plate and incubated at room temperature for five days.

Those isolates having only a few total differences (additional carbon sources utilized or carbon sources not utilized) from the wild-type were considered for further experimentation.

3.4.4 Growth comparison of the *gfp*+ isolates to *P. fluorescens* BRG100

Growth curves of the selected *gfp*+ isolates were prepared and compared to the wild-type to ensure no genes significantly involved in growth were interrupted by the

random gfp gene insertion. Based on the above criteria (BIOLOG and morphology), the following isolates were selected for this experiment: BRG*gfp*-5, 14, 15 and 16, as well as the wild-type, BRG100.

The following protocol was used to generate the growth curves. All isolates were grown on nutrient agar for 72 hours at room temperature. A single colony was picked from each plate and added to a sterile 50 ml centrifuge tube containing 20 ml nutrient broth. The tubes were placed on the rotary shaker at 150 rpm and 15ºC for 48 hours. The culture broth was then adjusted to an optical density of 0.4 at A_{600} and 100 µl of this adjusted broth was added to two 500 ml baffled flasks, one containing 100 ml nutrient broth and the other 100 ml M9 molasses media. The flasks were incubated on the rotary shaker at 150 rpm and 15ºC.

Every four hours following inoculation, optical density readings were taken for a total of 56 hours. The optical density was read using the spectrophotometer at A_{600}. The M9 molasses was diluted appropriately and plated using the Spiral Biotech Autoplate 4000 (Spiral Biotech, Bethesda, MD, USA) (**Figure 3.1**).

Figure 3.1. The Spiral Biotech Autoplate 4000 used to enumerate the wild-type and the 4 *gfp*+ isolates to generate growth curves.

All isolates were plated using the spiral plate counter on plate count agar (PCA) (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at room temperature for 48 to 72 hours. The plates were enumerated using a computerized program: CIA-BEN (See **Appendix E**). The doubling time for each isolate was then calculated using the formula:

T_d = In2 (or 0.693) / m

Where m was the slope of the line derived from the growth curve.

3.4.5 Comparison of the strength of fluorescence among *gfp*+ isolates

The 3 selected *gfp*+ isolates were grown in nutrient broth for 48 hours on the rotary shaker at 150 rpm. Wet mounts were then prepared and the isolates were viewed under the epifluorescent microscope at 40X and 100X oil immersion.

3.4.6 Comparison of root colonization ability in liquid broth

Based on the performance of strain BRG*gfp*-15, it was selected for further experiments. The following root colonization assay was used to enumerate of BRG100 and BRG*gfp*-15 bacteria on the roots of green foxtail and wheat seedlings. This was done to quantify the root colonization ability of BRG*gfp*-15 relative to the wild-type in order to determine no vital genes involved in the colonization process had been interrupted by the transposon mutagenesis.

Wild-type BRG100 and BRG*gfp*-15 isolates were streaked onto nutrient agar plates and incubated at room temperature for 48 hours. Each strain was inoculated into its own flask containing M9 molasses media and incubated on the rotary shaker at 150 rpm and 15 ºC for 48 hours. Two ml of culture broth was transferred to growth pouches containing green foxtail seedlings according to the growth pouch assay protocol described in section **3.4.2**.

Following an incubation period of 14 days the roots were removed from each of the ten green foxtail seedlings per growth pouch. The roots were placed in a Stomacher bag (Model 400 bag #6041) containing 100 ml of sterile phosphate buffer. They were mixed in the Seward Laboratory Blender Stomacher 400 (Seward, West Sussex, UK) for 20 minutes (**Figure 3.2**).

Figure 3.2. The Seward Laboratory Blender Stomacher 400 used to wash cells of the wild-type and BRG*gfp*-15 from green foxtail and wheat roots.

One ml aliquot of the phosphate buffer containing the suspended bacteria was removed from the Stomacher bag and diluted appropriately. The following dilutions were plated: 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. The plates were incubated for 48 hours and then enumerated using CIA-BEN. The roots and seeds of each of the seedlings were removed from the Stomacher bag and placed in a petri dish for 24 hours to get rid of excess moisture. The roots were weighed in order to obtain cfu/g dry root values.

3.4.6.1 Statistical analysis

The experimental design was a randomized complete block design. There were three treatments: the wild-type, untreated control, and BRG*gfp*-15. There were three replicates (growth pouches) and the experiment was conducted three times. Statistical analysis was performed with SAS 9.1 (English). The experiment was analyzed with

General Linear Model (GLM). The Student-Newman-Keuls (SNK) test was used to determine whether means were significantly different at a significance level of $p<0.05$.

3.5 Root colonization of green foxtail seedlings by *Pseudomonas fluorescens* BRG*gfp*-15

3.5.1 Root colonization assay in liquid broth

3.5.1.1 Enumeration of specific root sections

The objective of the above root colonization assay was to determine whether there was any significant difference between the ability of the wild-type and BRG*gfp*-15 to colonize the roots of green foxtail and wheat seedlings, and to obtain quantitative data on the number of bacteria per gram of root material. Once this had been determined, it was of interest to observe the root in sections in order to determine the spatial patterns of colonization of the bacteria.

In this assay, all aspects of the root colonization assay were performed according to the protocol described in section **3.4.6** with one major modification. Each seedling was measured with a small ruler and divided into three equal sections: the seed and proximal 1/3 root section, the middle 1/3 section, and the distal 1/3 section. The ten seedlings from each growth pouch were then placed into 3 different Stomacher bags: the first containing all ten seeds and proximal 1/3 root sections, the second all ten middle 1/3 root sections and the final ten distal 1/3. This allowed enumeration of the bacteria colonizing the specific sections and revealed if the bacteria had any preference for a certain region of the root.

3.5.1.1.1 Statistical analysis

The experimental design was a randomized complete block design. There were two treatments, an untreated control and BRG*gfp*-15. There were three growth pouch replicates, three plate replicates, and the experiment was conducted three times. Statistical analysis was performed with SAS 9.1 (English). The experiment was analyzed with GLM. SNK was used to determine whether means were significantly different at a significance level of $p<0.05$.

3.5.1.2 Analysis of roots using confocal and epifluorescent microscopy

Seedlings in growth pouches were also prepared as described above for observation under the epifluorescent and confocal microscopes. Three seedlings were randomly selected from each of the nine pouches and examined under the epifluorescent microscope. Seedlings were incubated for a minimum of 4 hours in nutrient broth at 50 rpm on the rotary shaker. The metabolism of BRG*gfp*-15 was increased by the added nutrients enabling optimized expression of gfp. Seedlings were removed from the broth and rinsed several times with sterile water to ensure only bacterial cells firmly adhering to the root were present. Each seedling was placed on its own microscope slide and its shoot was glued down using silicone adhesive and then submerged in distilled water. The seedlings were observed using a Nikon Type 104 epifluorescent microscope with a UV-1A filter at 40x magnification (Nikon Canada Inc., Mississauga, ON, Canada). A qualitative analysis was conducted to determine if there was any preference of the

bacteria to colonize specific areas of the root. Images were also obtained with a BioRad MRC1024 Lasersharp Scanning Confocal Laser Microscope to illustrate any trends that were found. The Biorad laser fluorescence scanner were mounted on a Nikon Microphot SA equipped with either a 63x Zeiss water immersible NA 0.9 or a 10x Nikon water immersible NA 0.3. All images were obtained using the following filter sets: excitation/ emission 488/522 +/- 20 nm, 568/605 +/- 20 nm, and 647/680 +/- 20 nm.

3.5.2 Root colonization assay using pesta granules

3.5.2.1 Formulation of BRG*gfp*-15 into pesta granules

BRG *gfp*-15 was formulated into pesta granules according to the AAFC protocol, as follows. Strain BRG*gfp*-15 was streaked onto a nutrient agar plate and incubated at room temperature for 48 hours. An isolated BRG*gfp*-15 was then transferred to a 500 ml flask containing 200 ml of sterile M9 molasses media. The flask was then placed on the rotary shaker and incubated at 15ºC and 150 rpm for 64 hours.

The dry ingredients were weighed out and packaged: 300 g oat flour, 80 g maltose, and 20 g peat. To make the pesta dough, 1 package of ingredients was placed in the Viking Professional food processor (Viking, Greenwood, MS, USA). One hundred and seventy ml of culture broth containing BRG*gfp*-15 at a concentration of approximately 10^7 viable cells/gram was gradually added while the processor was mixing at the maximum power of 1,000 watts **(Figure 3.3**). Processing was complete when the dough was uniformly mixed (1-2 minutes).

Figure 3.3. The Viking Professional food processor used to mix the dry ingredients of pesta with the liquid bacterial culture.

The dough was extruded through the MG55 Granulator (LCI Corporation, Charlotte, NC, USA), with an orifice size of 1.2 mm (**Figure 3.4**). As the dough was being extruded, it was manually scraped off the mesh basket into the attached pan.

Figure 3.4. The MG55 Granulator used to extrude pesta.

Each 400 g batch was divided into two 200 g batches, which was then transferred to the Spheronizer (LCI Corporation, Charlotte, NC, USA), with a 1 mm friction plate, operated at 1,000 rpm for 2 minutes (**Figure 3.5)**, which resulted in pesta granules of 1 mm diameter.

Figure 3.5. The Spheronizer used to reduce pesta into granules of a uniform size.

Each batch was then transferred to a cookie sheet and placed in the laminar flow cabinet (Forma Scientific, Marietta, OH, USA) to dry for 1 hour, and then transferred to a dryer tub and placed on the Sherwood Scientific fluidized bed drier (Sherwood Scientific Ltd., Cambridge, UK) maintained at 42.5ºC, with a blower speed of 10, for 20 minutes (**Figure 3.6**).

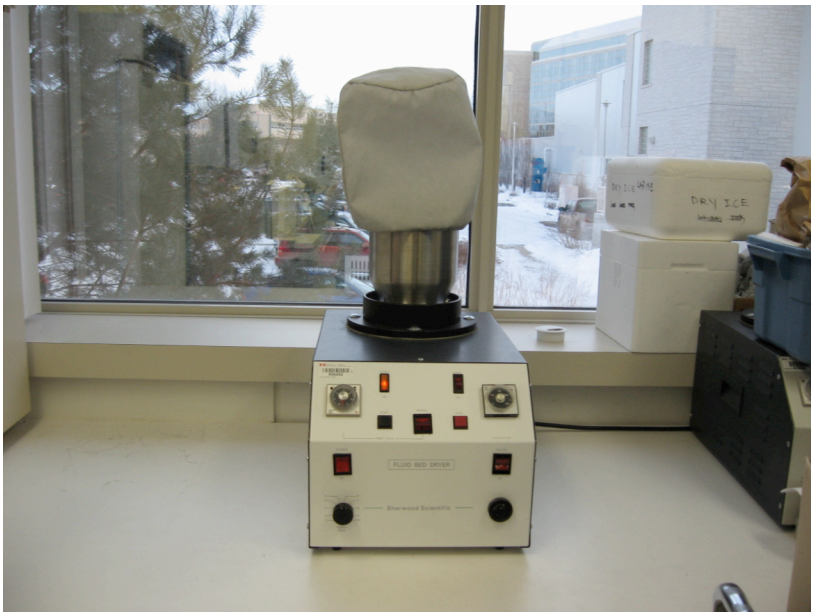


Figure 3.6. The Sherwood Scientific fluidized bed drier used to remove excess moisture from the pesta granules.

The 400 g batch was then deposited into a clean plastic container.

3.5.2.2 Water activity of pesta granules

After 24 hours of refrigeration at 4ºC in the sealed container the water activity (A_w) was measured. The pesta was then returned to storage in the plastic container at 14ºC.

3.5.2.3 Bacterial enumeration of pesta

Following 48 hours of storage, the pesta was enumerated to ensure a concentration of approximately 10^8 viable cells/gram. The protocol was as follows. Ten grams of pesta granules were added to a dilution bottle containing 90 ml sterile Butterfield's phosphate-buffer stock solution (10^{-1}). The pesta was left to soak for 2

minutes. It was then homogenized at 15,000 rpm for 20 seconds with the Heidolph DIAX 900 homogenizer with a 20 mm diameter saw-tooth generator probe (Rose Scientific Ltd., Edmonton, AB**,** Canada)**.** The mixture was allowed to settle for 3 minutes and diluted using Butterfield's phosphate-buffer stock solution to 10^{-4} and 10^{-5}. The suspension was then plated using the Spiral Biotech Autoplate 4000 on PCA plates. Two 10 g samples were plated and 3 plate replicates were made for each sample. The plates were then enumerated using the computerized program CIA-BEN.

3.5.2.4 Enumeration of specific root sections

BRG*gfp*-15 was formulated into pesta granules at a concentration of 10^7 viable cells/gram as described above. Growth pouches seeded with green foxtail were inoculated with pesta according to the AAFC protocol. The treatments included BRG*gfp*-15 and an untreated control, which was pesta containing no bacteria. The protocol was similar to that using liquid broth culture discussed in section **3.5.1.1**, but with a few major changes explained below.

Growth pouches were seeded with 15 green foxtail seeds. One gram of pesta was spread evenly over the trough and seeds. Approximately 1 g of sterile Redi Earth (Sun Grow Horticulture, see **Appendix I**) was placed over the pesta. Twenty ml of sterile water was added to each pouch and the pouches were incubated for 14 days under the same conditions outlined in the growth pouch bioassay described in section **3.4.2**.

Following incubation of green foxtail growth pouches, localization of BRG*gfp*-15 on the different plant root sections was determined by dilution plating according to the protocol outlined above in the root colonization in liquid broth assay.

3.5.2.4.1 Statistical analysis

The experimental design was a randomized complete block design. There were two treatments: an untreated control and BRG*gfp*-15; three growth pouch replicates; three plate replicates; and the experiment was conducted three times. Statistical analysis was performed with SAS 9.1 (English). The experiment was analyzed with GLM. SNK was used to determine whether means were significantly different at a significance level of $p<0.05$.

3.6 Effect of soil moisture, temperatures, and type on BRG*gfp*-15 using the thermogradient plate.

3.6.1 Determination of soil characteristics

Soils were collected from Saskatoon AAFC research farm and Scott AAFC research farm and were analyzed by the ALS Laboratory Group (819 58 Street East Saskatoon, SK, Canada) for pH and overall soil texture. The amounts of available potassium, nitrogen, sulfur, and phosphorus were also determined.

3.6.2 Sterilization of soils

Soils from Saskatoon and Scott were sterilized as follows: soils were placed in large, wide-bottomed flasks at 150 g/flask. They were autoclaved with the Amsco Century SV-120 Scientific Prevacuum Sterilizer (Steris Corporation, Mentor, OH, USA) on the gravity cycle for 40 minutes. They were then placed in the Fisher Isotemp oven, senior model (Fisher Scientific Company, Ottawa, ON, Canada) at 101ºC for no less than 24 hours and sterile water was added to bring the moisture up to the appropriate percent of field capacity as outlined below.

3.6.3 Determination of soil moisture holding capacity

Soil moisture holding capacity was determined using the following procedure. One hundred and fifty grams of soil was autoclaved and then placed in the dry oven at 101ºC for no less than 24 hours. The soil was then removed and weighed. One hundred grams of this sterile dry soil was then placed in a funnel and held over a flask. One hundred mls of sterile water was added in very small increments to the soil. The first drop of water that came out the bottom of the funnel signified the moisture holding capacity. This procedure was performed three times.

3.6.4 Effect of soil moisture, temperatures, and type on BRG*gfp*-15 using the thermogradient plate.

Soils from both locations were inoculated with pesta containing BRG100*gfp*-15 at a concentration of approximately 10^7 viable cells/g. The soils were placed in petri dishes that maintain specific moisture level on a thermal gradient apparatus, which has precise temperature control **(Figure 3.7**).

The temperatures that were used were between 5 and 35ºC to represent soil temperatures of spring and summer in Canadian prairies. Three temperature regimes were used with the temperature cycling from maximum temperature to minimum temperature then back up to maximum over a 24-hour period. The three temperature regimes were: 15/5, 35/25, and 25/15ºC. Three moisture levels were used as well: 25%, 50%, and 75% of soil moisture holding capacity for each soil type. There were three temperature regimes, three moisture levels, and two soil types, Scott and Saskatoon for a total of 18 treatments.

To each plate, 35 g of soil was evenly spread over the bottom of the plate. Then the pesta was placed in a furrow on top. Each plate contained a total of 1 gram of pesta granules evenly spaced in one row lengthwise. Pesta granules were removed from each plate at 2 weeks, 4 weeks, and 6 weeks and plated with the Spiral Biotech Autoplate 4000 and enumerated using CIA-BEN to determine the survival of the bacteria in the pesta granules over time and under the different temperatures, moistures, and soil types. At each time interval, 0.2 grams of pesta granules were removed and added to a dilution blank containing 1.8 mls phosphate buffer. The tube was vortexed on high speed for 30 seconds and allowed three minutes to settle out. Dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were made and enumerated.

Figure 3.7. The thermogradient plate apparatus used for the incubation of BRG*gfp*-15 over a 42 day period.

Some plates were contaminated with microorganisms other than BRG*gfp*-15, therefore they required manual counts with the Bantex colony counter 920A (American Bantex Corp., Burlingame, CA, USA) (**Figure 3.8**).

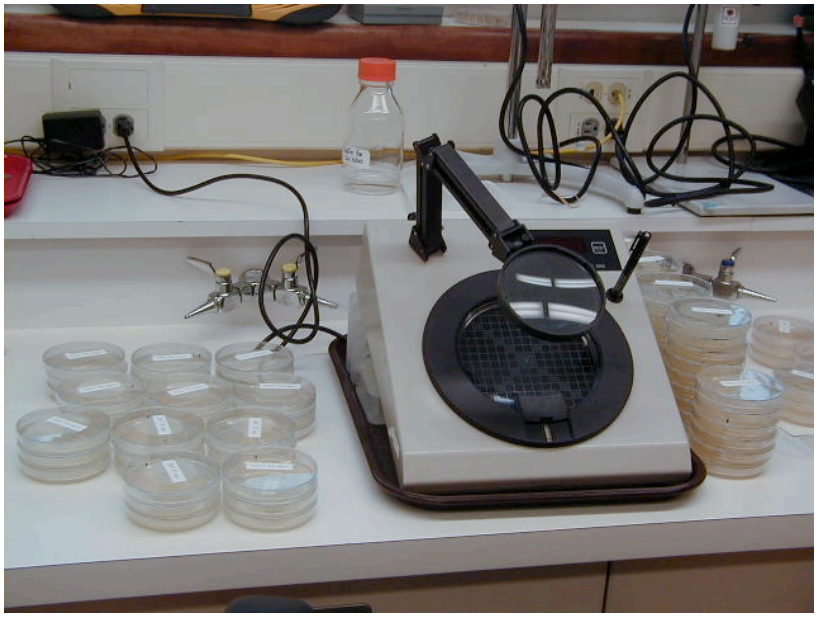


Figure 3.8. The Bantex colony counter 920A used to obtain manual counts of BRG*gfp*-15.

3.6.4.1 Statistical analysis

The experimental design used was a 3x3x2x3 factorial randomized complete block design. There were 3 temperature regimes: 5-15ºC, 15-25ºC, and 25-35ºC; 3 moisture levels: 25, 50, and 75% of soil moisture holding capacity; and two soil types (Saskatoon: clay and Scott: clay loam) for a total of 18 treatments. There were 4 thermogradient cell replicates and 3 plate replicates. Samples were taken at 3 time intervals: 14, 28, and 42 days. Treatments were randomized within the plate and the

experiment was conducted twice. Data was analyzed using SAS 9.1 (English). The analysis was done with GLM. SNK was used to determine whether means were significantly different at a significance level of $p<0.05$.

4 Results

4.1 Construction of *gfp* mutants

Pseudomonas fluorescens BRG100 was transformed as described in section **3.3**. Out of a total of 100 cfu/plate, 12-18 *gfp*+ colonies were found on each of six kanamycin plates. Sixteen of the brightest *gfp*+ colonies were selected and subcultured. The sixteen plates were confirmed using long-wave UV light to contain only *gfp*+ colonies One *gfp*+ isolate was then isolated from each plate and frozen in the ultra low temperature freezer as described in section **3.2**.

4.2 Selection of the *gfp*+ mutant strain

4.2.1 Comparison of morphology

Plates containing each *gfp*+ isolate and the wild-type, BRG100, were examined under the dissecting microscope. All sixteen isolates formed colonies that had identical morphological characteristics to the wild-type, including colour, which was creamy white, form, which was round, elevation, which was convex, and margin, which was smooth.

4.2.2 Comparison of biocontrol efficacy: Growth pouch assay

The *gfp*+ isolates were compared to the wild-type for their biological control efficacy of green foxtail (**Table 4.1**). The results were converted to % root suppression according to the following formula:

untreated control (root length mm) – BRG*gfp* isolate (root length mm) / untreated control (root length mm) * 100 = % root suppression

Table 4.1. Comparison of the ability of BRG100 and the 16 *gfp*+ isolates to suppress the growth of green foxtail roots.

Isolate	% Root Suppression
BRG*gfp*-1	10.3 f
BRG*gfp*-2	77.0 abc
BRG*gfp*-3	67.7 e
BRG*gfp*-4	69.5 cde
BRG*gfp*-5	79.0 ab
BRG*gfp*-6	77.8 abc
BRG*gfp*-7	0.0 g
BRG*gfp*-8	73.5 abcd
BRG*gfp*-9	72.8 abcd
BRG*gfp*-10	67.7 de
BRG*gfp*-11	75.5 abcd
BRG*gfp*-12	71.7 bcd
BRG*gfp*-13	78.3 abc
BRG*gfp*-14	80.2 ab
BRG*gfp*-15	81.7 a
BRG*gfp*-16	78.2 abc
BRG100(2)WT	81.5 a

*Means with the same letter are not significantly different according to t test. Significance level is 0.05%.

The wild-type, BRG100, had 82 % root suppression. Following statistical analysis with SAS, ten isolates were found to be sufficiently similar to the wild-type in their ability to suppress the root growth of green foxtail seedlings and would be used in further studies. These isolates included BRG*gfp*-2 with 77% root suppression, BRG*gfp*-5 with 79%, BRG*gfp*-6 with 77%, BRG*gfp*-8 with 74%, BRG*gfp*-9 with 73%, BRG*gfp*-11 with

76%, BRG*gfp*-13 with 78%, BRG*gfp*-14 with 80%, BRG*gfp*-15 with 82%, and BRG*gfp*-16 with 78%. The seven isolates most similar to the wild-type out of these ten were chosen for further study. The remaining six isolates had significantly reduced biocontrol efficacy. BRG*gfp*-3 had 68% root suppression, BRG*gfp*-4 had 70%, BRG*gfp*-10 had 68%, and BRG*gfp*-12 had 72% root suppression. BRG*gfp*-1 had 10% root suppression and BRG*gfp*-7 showed 0% root suppression (**Table 4.1)**.

4.2.3 Comparison of carbon utilization

Carbon utilization profiles were generated for each *gfp*+ isolate and the wild-type BRG100, enabling a comparison of the ability to utilize the various carbon sources. Differences between the wild-type and each of the 16 *gfp*+ isolates are reported in **Table 4.2**. This included the number of carbon sources that were utilized by the wild type but not by that particular *gfp*+ isolate, as well as the number of carbon sources that were utilized by the *gfp*+ isolate that were not utilized by the wild-type.

Table 4.2 A comparison of the ability of BRG100 and the 16 *gfp*+ isolates to utilize carbon sources.

Isolate	C sources not utilized	Additional C sources utilized	Total differences
BRG*gfp*-1	10	0	10
BRG*gfp* -2	12	1	13
BRG*gfp*- 3	10	1	11
BRG*gfp*- 4	20	1	21
BRG*gfp*- 5	1	0	1
BRG*gfp*- 6	10	0	10
BRG*gfp* -7	5	1	6
BRG*gfp*- 8	1	0	1
BRG*gfp*- 9	8	0	8
BRG*gfp*- 10	6	0	6
BRG*gfp* -11	5	0	5
BRG*gfp*- 12	1	0	1
BRG*gfp*- 13	13	0	13
BRG*gfp*- 14	1	1	2
BRG*gfp* -15	2	0	2
BRG*gfp* -16	2	1	3

*Column 2 represents the number of carbon sources utilized by the wild-type but not the *gfp*+ isolate, column 3 represents the number of carbon sources utilized by the *gfp*+ isolate but not by the wild-type, and column 4 represents the total number of differences (column 2 + column 3).

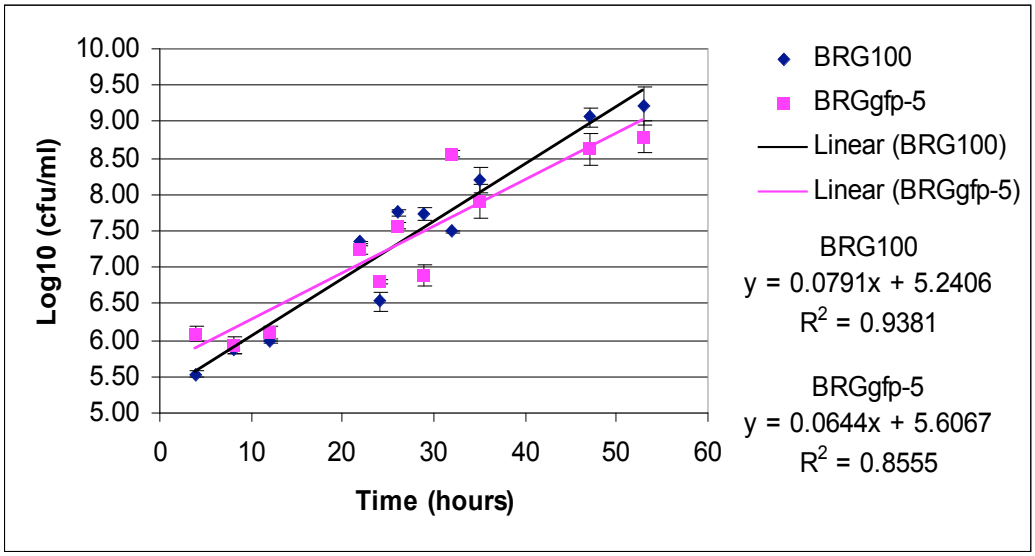
Of the seven isolates selected on the basis of biological control efficacy (BRG*gfp*-2, 5, 6, 13, 14, 15, and 16) the following isolates were sufficiently close to the wild-type in their ability to utilize the 95 carbon sources in the BIOLOG plate: BRG*gfp*-5, 14, 15, and 16. BRG*gfp*-5 had only 1 difference; it did not utilize sucrose, which was utilized by the wild-type. BRG*gfp*-14 had 2 differences. It could not utilize α-keto valeric acid as could the wild-type, but it was able to utilize succinamic acid, which the wild-type was unable to. BRG*gfp*-15 had 2 differences as well. It was unable to utilize α-D-lactose and α-keto valeric acid, both of which were utilized by the wild-type. BRG*gfp*-16 had 3 differences. It was unable to utilize sucrose and D, L-lactic acid, which were utilized by the wild-type, but was able to utilize succinamic acid, which was not utilized by the wild-type. These four isolates were used in the subsequent growth curve assay.

4.2.4 Growth comparison of *gfp*+ isolates

Growth curves of the wild-type, BRG100, and the 4 selected *gfp*+ isolates, BRG*gfp*-5, 14, 15, and 16 are as follows:

The growth of BRG*gfp*-5 and the wild-type BRG100 in the M9 molasses media as determined by spiral plating is shown in **Figure 4.1a** and in nutrient broth as determined by optical density readings is shown in **Figure 4.1b**. Direct counts revealed a doubling time of 8.7 hours for the wild-type and 10.8 hours for BRG*gfp*-5. Optical density readings revealed that for both isolates log phase began at 15 hours and ended at 26 hours.

a)



b)

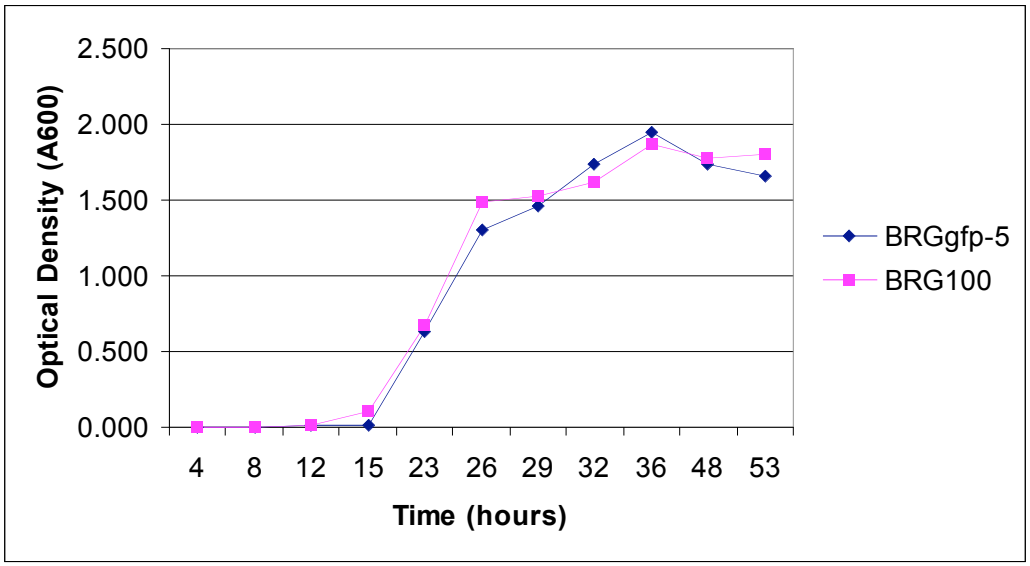
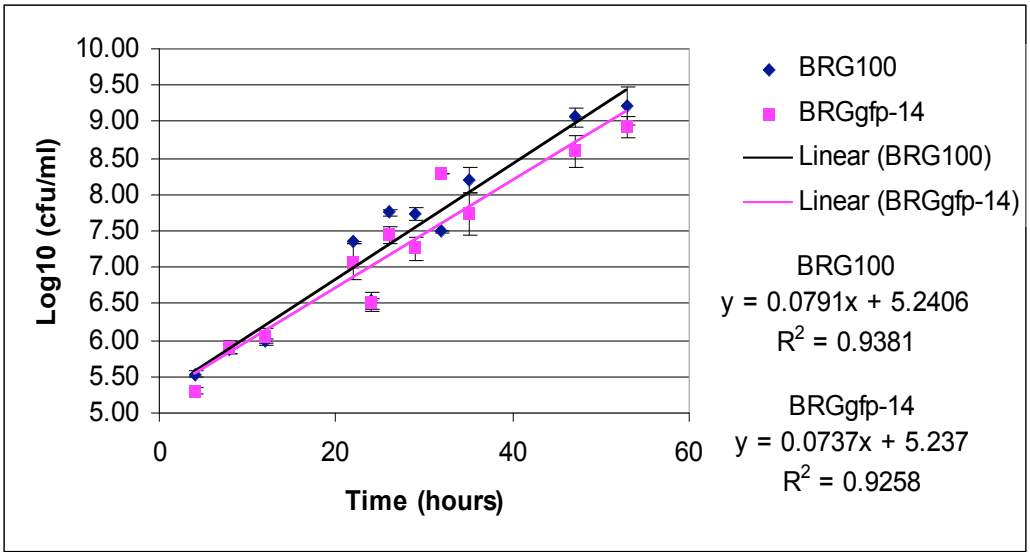


Figure 4.1. The growth curves of BRG100 and BRG*gfp*-5 grown in a) M9 molasses media and enumerated with the spiral plater and b) nutrient broth and enumerated by optical density readings with the spectrophotometer.
Error bars represent the standard error of the mean. (Note: The standard error was too low on many of the points to be seen)

The growth of BRG*gfp*-14 and the wild-type, BRG100, as determined by spiral plating is shown in **Figure 4.2a** and by optical density readings is shown in **Figure 4.2b**. Direct counts revealed that the doubling time for BRG*gfp*-14 was 9.4 hours. Optical density readings revealed that BRG100 and BRG*gfp*-14 began log phase at 15 hours. BRG100 ended log phase at 26 hours, while log phase did not end until 32 hours for BRG*gfp*-14.

a)



b)

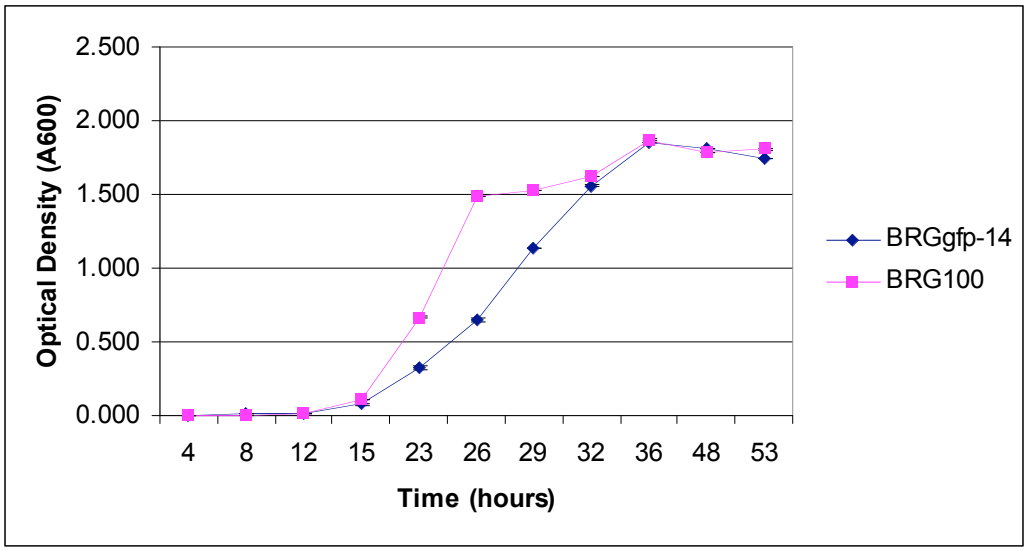
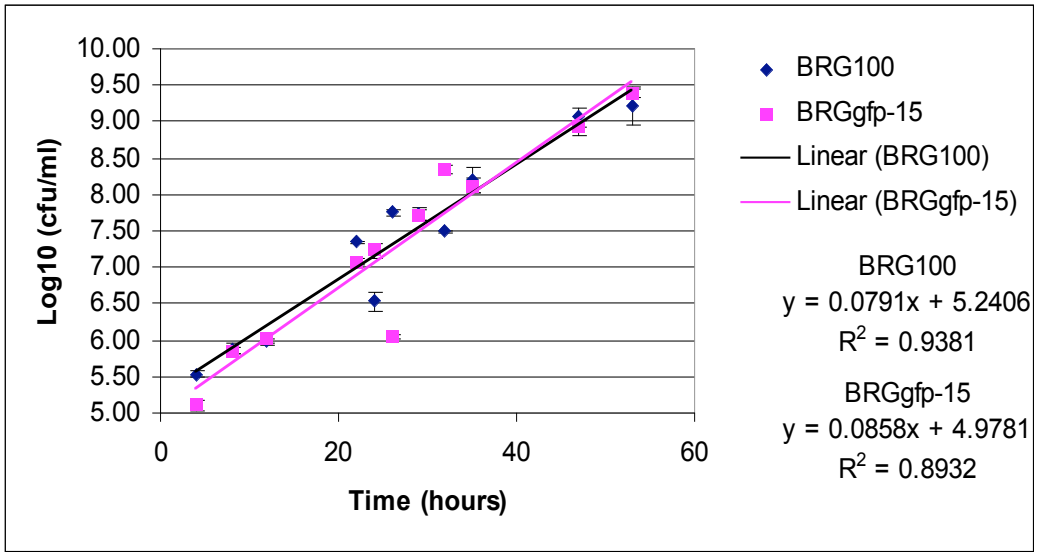


Figure 4.2. The growth curves of BRG100 and BRG*gfp*-14 grown in a) M9 molasses media and enumerated with the spiral plater and b) nutrient broth and enumerated by optical density readings with the spectrophotometer.
Error bars represent the standard error of the mean. (Note: The standard error was too low on many of the points to be seen).

The growth of BRG*gfp*-15 and the wild-type, BRG100, as determined by spiral plating is shown in **Figure 4.2a** and by optical density readings is shown in **Figure 4.2b**. Direct counts revealed that the doubling time for BRG*gfp*-15 was 8.1 hours. Optical density readings revealed that log phase for BRG100 and BRG*gfp*-15 began at 15 hours, and ended at 26 hours.

a)



b)

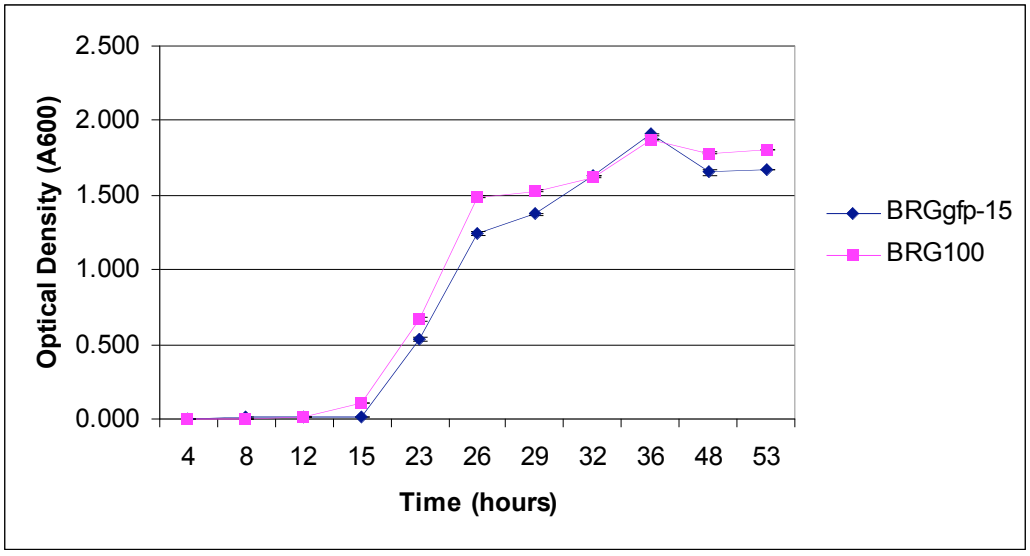
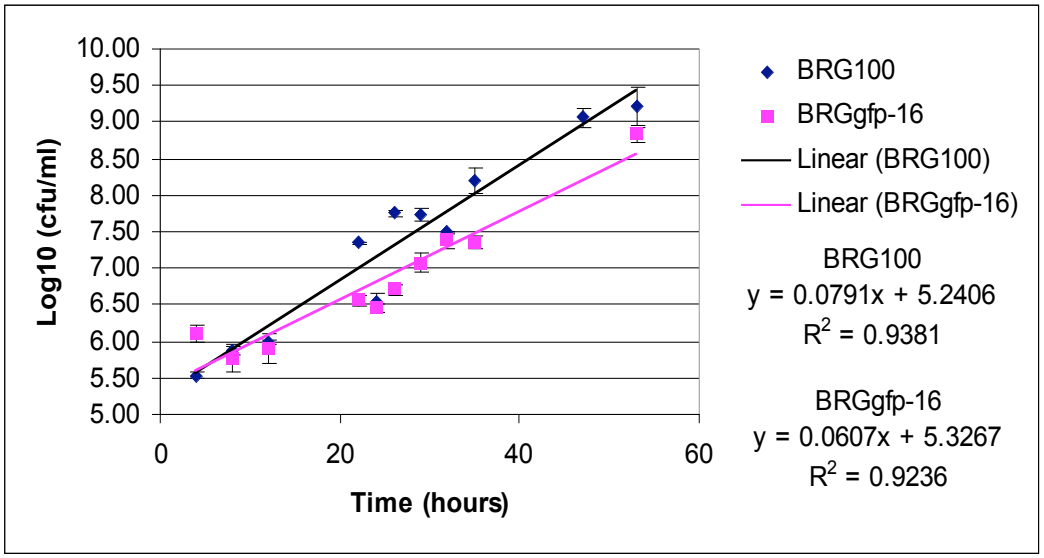


Figure 4.3. The growth curves of BRG100and BRG*gfp*-15 grown in a) M9 molasses media and enumerated with the spiral plater and b) nutrient broth and enumerated by optical density readings with the spectrophotometer.
Error bars represent the standard error of the mean. (Note: The standard error was too low on many of the points to be seen).

The growth of BRG*gfp*-16 and the wild-type, BRG100, as determined by spiral plating is shown in **Figure 4.4a** and by optical density readings is shown in **Figure 4.4b**. Direct counts revealed that the doubling time for BRG*gfp*-16 was 11.4 hours. Optical density readings revealed that BRG100 reached log phase at 15 hours and ended at 26 hours. BRG*gfp*-16 did not reach log phase until 29 hours and ended log phase at 48 hours.

a)



b)

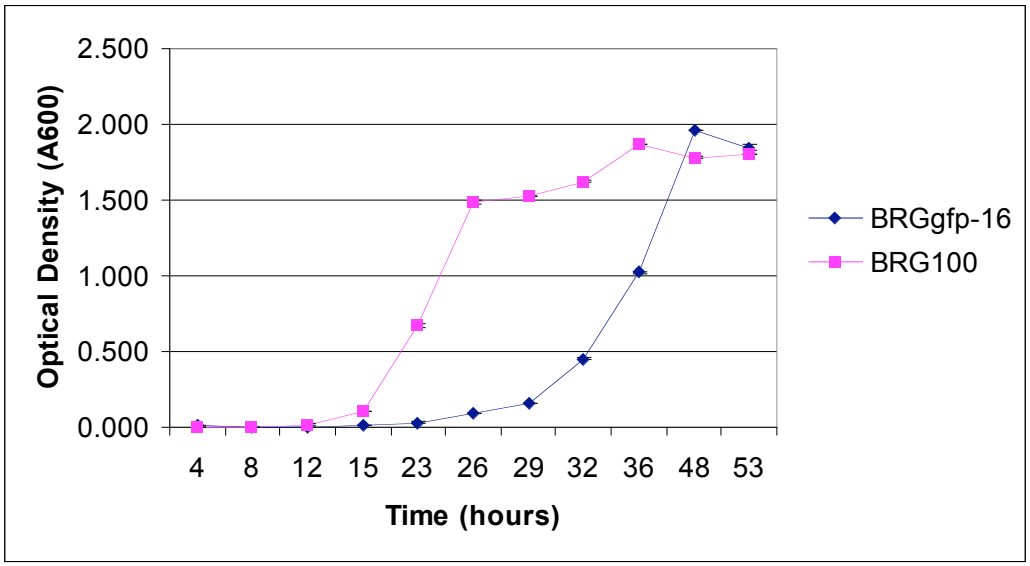


Figure 4.4. The growth curves of BRG100 and BRG*gfp*-16 grown in a) M9 molasses media and enumerated with the spiral plater and b) nutrient broth and enumerated by optical density readings with the spectrophotometer.
Error bars represent the standard error of the mean. (Note: The standard error was too low on many of the points to be seen)

4.2.5 Comparison of strength of fluorescence

BRG*gfp*-5, 14, and 15 all were sufficiently close to the wild-type, BRG100, in biological control efficacy, carbon utilization, and rate of growth. BRG*gfp*-15 was chosen out of these three *gfp*+ isolates in part because it emitted the strongest fluorescence (**Figure 4.5**).

a) b)

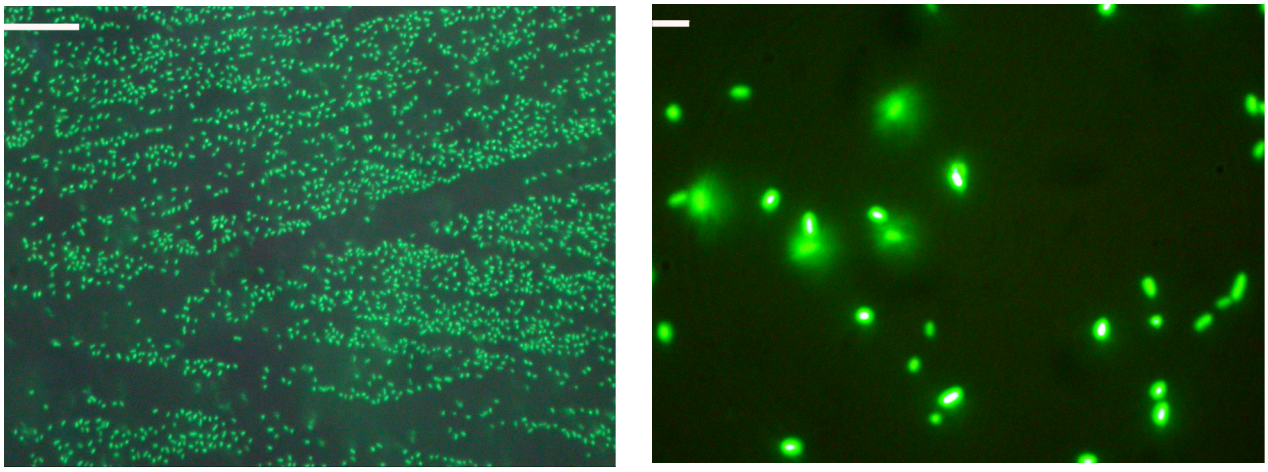


Figure 4.5. Wet mount of BRG*gfp*-15 with a a) 40x objective and a b) 100X objective (oil immersion) using epifluorescent microscopy.
White scale bars represent 100µm for a) and 10µm for b).

4.2.6 Comparison of root colonization ability in liquid broth

Comparison of the root colonizing ability between the wild-type, BRG100, and BRG*gfp*-15 was conducted to ensure BRG*gfp*-15 would be a suitable *gfp*+ isolate for use in the remainder of the experiments. The total number of colony forming units that had

colonized green foxtail and wheat roots was determined for both the wild-type, BRG100, and BRG*gfp*-15. The results were reported as cfu/ml and then converted to both cfu/gram dry root weight and the $\log_{10}$ value (**Table 4.3**). This was also performed with an untreated control (M9 molasses media with no bacteria). No bacteria were observed from washings of the root, confirming that there were no other bacteria present on the seedlings.

Table 4.3. Comparison of colonization by BRG100 and BRG*gfp*-15 of green foxtail and wheat roots following 14 days of incubation in growth pouches.

Plant	Isolate	Experiment	Log10 cfu/g	Letter grouping
Wheat	**BRG100**	1	7.54	
		2	7.46	
		3	7.43	
		Mean	**7.48**	**A**
	BRG*gfp*-15	1	7.67	
		2	7.58	
		3	7.33	
		Mean	**7.53**	**A**
Green Foxtail	**BRG100**	1	8.01	
		2	8.75	
		3	7.09	
		mean	**7.95**	**A**
	BRG*gfp*-15	1	8.27	
		2	8.04	
		3	6.79	
		mean	**7.7**	**A**

*Means with the same letter are not significantly different, as determined by analysis with SNK. Significance level was 0.05%.

On green foxtail roots, BRG100 was found to have a mean cfu/g dry root weight of $\log_{10}7.95$. BRG*gfp*-15 had a mean cfu/g dry root weight of $\log_{10}7.70$. There was no significant difference between the number of BRG*gfp*-15 cells and the number of wild-

type cells colonizing the roots of green foxtail seedlings (**Figure 4.6**). On wheat roots BRG100 was found to have a mean cfu/g dry root weight of $\log_{10}7.48$. BRG*gfp*-15 had a mean cfu/g dry root weight of $\log_{10}$ 7.53. There was no significant difference between the number of BRG*gfp*-15 cells and the number of wild-type cells colonizing the roots of wheat seedlings (**Figure 4.6).** BRG*gfp*-15 was considered suitable for the following experiments.

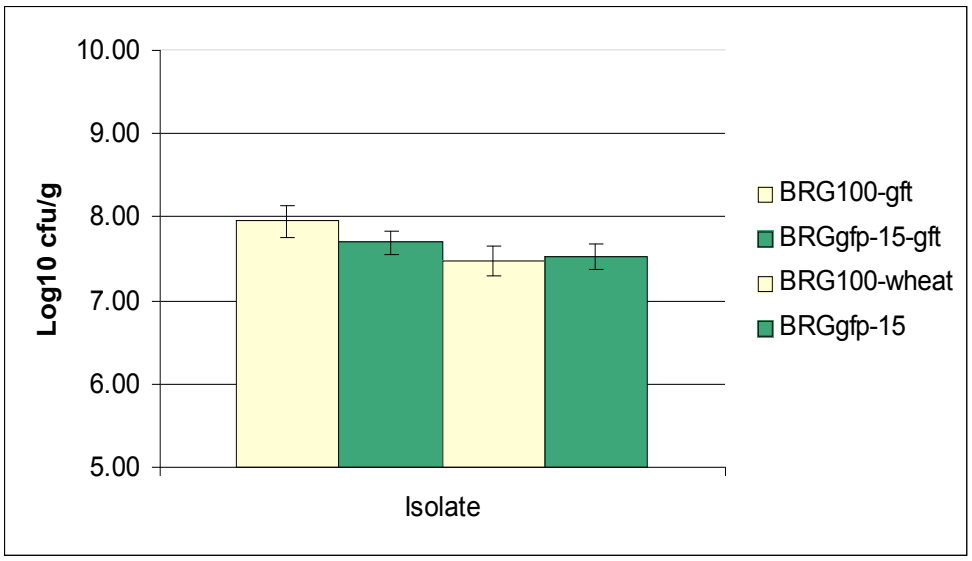


Figure 4.6. Colonization on green foxtail and wheat roots by BRG100 and BRG*gfp*-15 following 14 days of incubation in growth pouches.
Error bars represent the standard error of the mean

4.3 Root colonization of green foxtail seedlings by *Pseudomonas fluorescens* BRG*gfp*-15

4.3.1 Root colonization in liquid broth

4.3.1.1 Enumeration of specific root sections

The number of BRG*gfp*-15 cells colonized on the specific sections the roots of green foxtail seedlings was determined. The root was divided lengthwise into three equal parts. The first section is the proximal 1/3 section along with the seed, the middle 1/3 section and the distal 1/3 section. The results were obtained as cfu/ml and then converted to cfu/gram root dry weight and its $\log_{10}$ value (**Table 4.4**). This was also performed with an untreated control (M9 molasses media with no bacteria), and no bacteria were found from washings of the root.

Table 4.4. BRG*gfp*-15 colonization on the three lengthwise sections of green foxtail roots following 14 days of incubation in growth pouches.

Section	Experiment	Cfu/ml	Cfu/g	Log10 cfu/g	Letter grouping
Proximal	1	1.53E+06	8.76E+08	8.94	
	2	1.84E+06	1.04E+09	9.02	
	3	2.77E+06	1.61E+09	9.21	
	mean	**2.05E+06**	**1.17E+09**	**9.06**	**A**
Middle	1	1.24E+05	1.22E+08	8.09	
	2	3.11E+04	2.74E+07	7.44	
	3	1.47E+04	1.19E+07	7.07	
	mean	**5.65E+04**	**5.39E+07**	**7.53**	**B**
Distal	1	9.38E+03	1.30E+07	7.11	
	2	4.89E+03	7.20E+06	6.86	
	3	1.57E+04	1.14E+07	7.06	
	mean	**1.00E+04**	**1.05E+07**	**7.01**	**B**
	total	**2.11E+06**	**1.24E+09**	**9.09**	

*Means with the same letter are not significantly different, as determined by analysis with SNK. Significance level was 0.05%.

The proximal section had $\log_{10}9.06$ viable cells/g dry root weight, while the middle and distal sections had much less bacteria, $\log_{10}7.53$ and $\log_{10}7.01$, respectively. Statistical analysis revealed a significant difference between the proximal 1/3 section and seed as compared to the middle and distal sections (**Figure 4.7**). There was no significant difference between the middle and lower sections of the root.

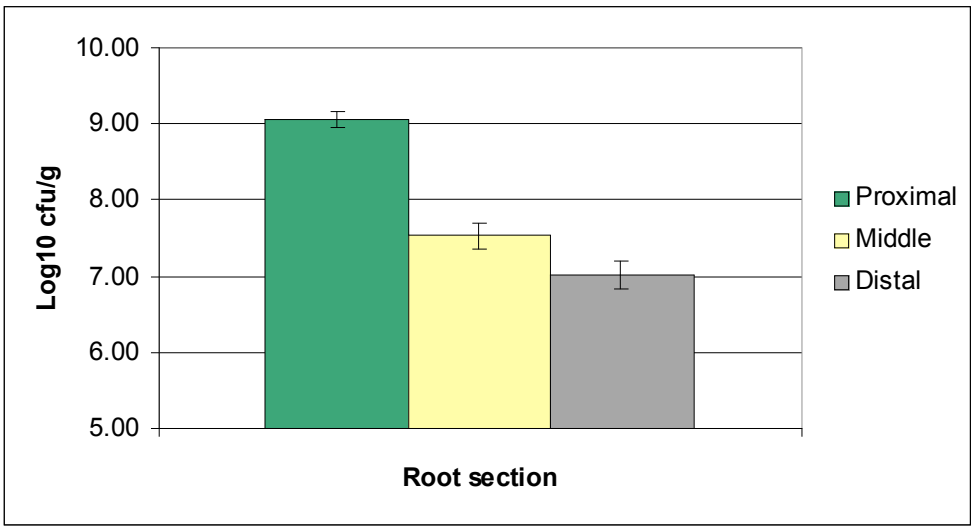


Figure 4.7. BRG*gfp*-15 colonization on the three lengthwise sections green foxtail roots following 14 days of incubation in growth pouches.
Error bars represent the standard error of the mean

4.3.1.2 Observation of roots under the confocal and epifluorescent microscopes

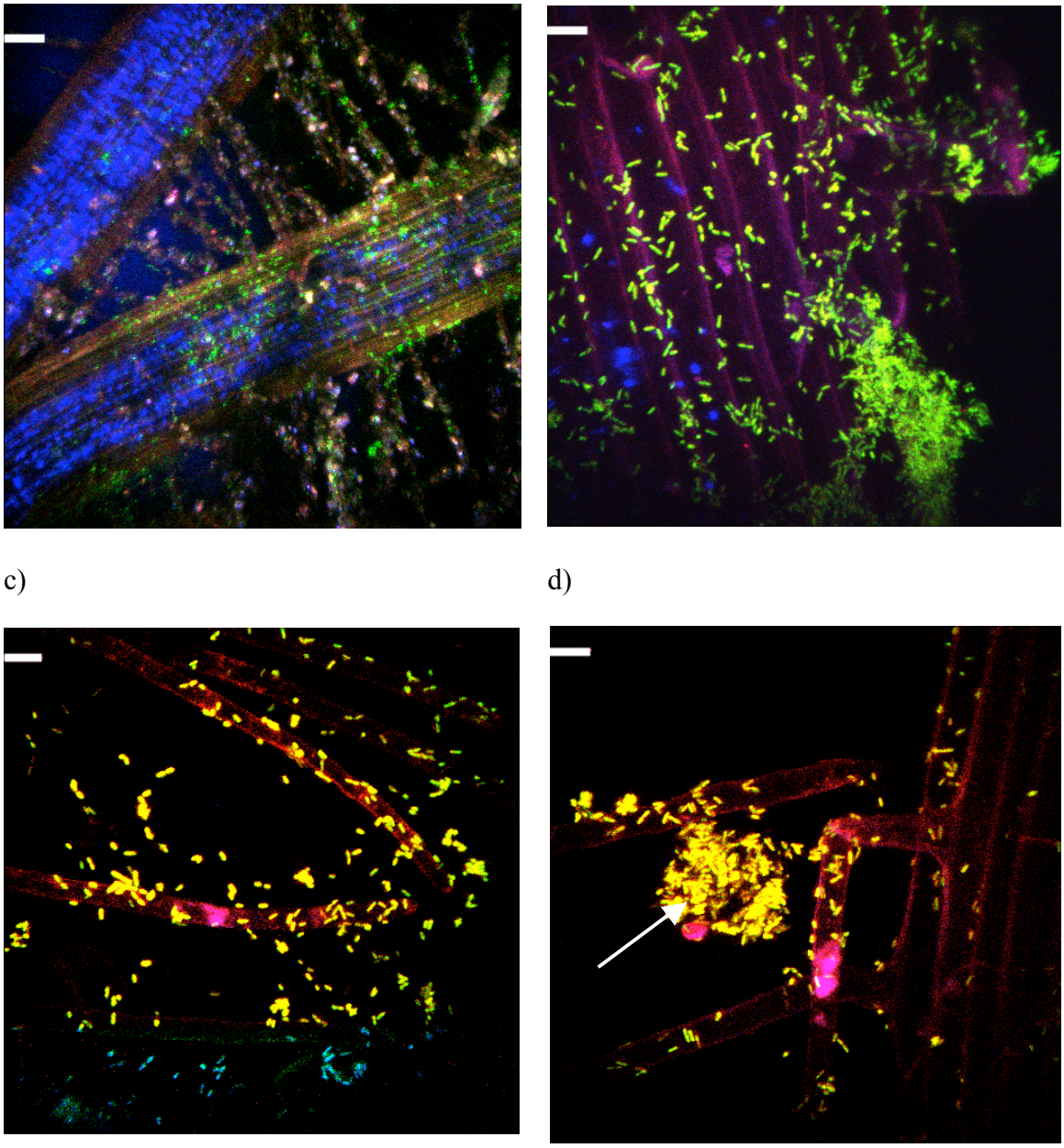
A total of 27 seedlings were observed under the epifluorescent and confocal microscopes (see **Appendix G**)**.** The different sections of each root were examined to determine if BRG*gfp*-15 had a preference for a section of the root. All three sections, the

proximal 1/3 section + seed, middle 1/3 section, and the distal 1/3 section, were heavily colonized **(Figure 4.8a**). Some seedlings had heavier colonization in the proximal section, while some were more heavily colonized in the middle or distal section. It was not obvious that the bacterium had a preference for the proximal section + seed.

Cells of BRG*gfp*-15 were present in both microcolonies and single cells spread evenly over the root (**Figure 4.8b**). This was observed in all three sections of the root, the proximal 1/3 section and seed, the middle 1/3 section, and the distal 1/3 section. There was no clear pattern of cells in any of the three sections of the root.

It was also observed that root hairs were always very heavily colonized in all three sections of the root. Root hairs from both the proximal and middle sections were always very heavily colonized (**Figure 4.8c** and **Figure 4.8d**). The cells were found both in single cells spread evenly on the hairs and also in microcolonies (note white arrows indicating the location of microcolonies). Sometimes one or more large microcolonies were found attached to a root hair (**Figure 4.8c**).

a) b)

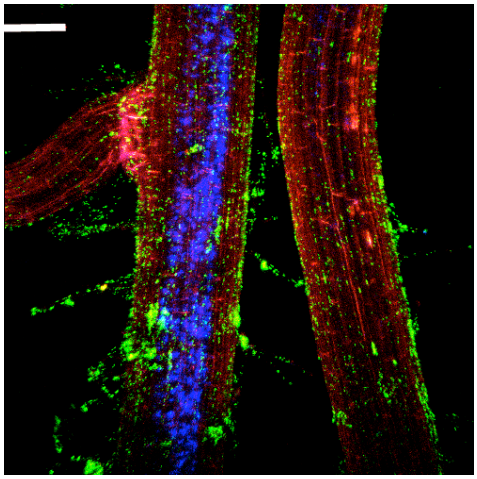


c) d)

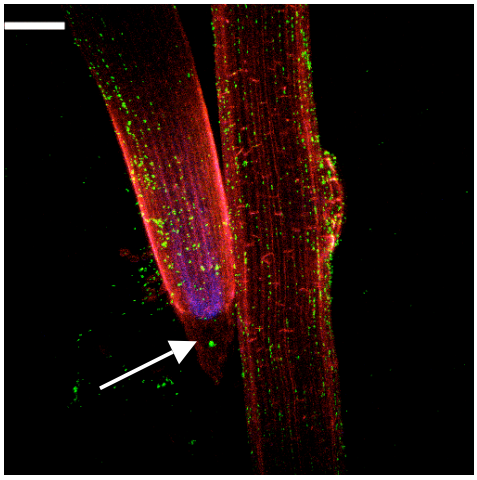
Figure 4.8. BRG*gfp*-15 on the root of a green foxtail seedling observed using confocal microscopy.
a) 10x Nikon water immersible NA 0.3; b) c) and d) 63x Zeiss water immersible NA 0.9 *White bar represents 100µm for a) and 10µm for b) c) and d). Note: blue in a) and b) is autofluorescence from chloroplasts; white arrows indicate the location of BRG*gfp*-15 microcolonies; BRG*gfp*-15 cells appear yellow in c) and d) due to excitation and emission wavelength used.

The distal1/3 section of the root was usually well colonized. However, even on seedlings that were not as heavily colonized in the distal section there was often a concentration of cells and microcolonies at or near the root tip [(**Figure 4.9a** and **Figure 4.9b**) (note white arrow indicating location of microcolony)]. Heavy colonization near the end of the root (~5mm from root tip) and at the root tip was often found. Heavy colonization of the seed was often observed, usually on the ventral portion of the seed (**Figure 4.9c**).

a)



b)



c)

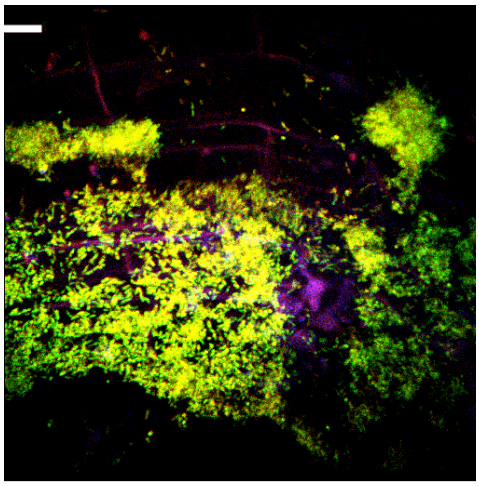


Figure 4.9. BRG*gfp*-15 on the root of a green foxtail seedling observed using confocal microscopy.
a) and b) 10x Nikon water immersible NA 0.3; c) 63x Zeiss water immersible NA 0.9. *White scale bars indicate 100µm for a) and b) and 10µm for c). Note: blue in a) is autofluorescence from chloroplasts; white arrow in b) indicates location of BRG*gfp*-15 microcolony at root tip; BRG*gfp*-15 cells appear yellow in c) due to excitation and emission wavelength used.

4.3.2 Root colonization using pesta granules

4.3.2.1 Formulation of BRG*gfp*-15 into pesta granules

BRG*gfp*-15 was formulated into pesta granules. An untreated control (no bacteria) was also prepared.

4.3.2.2 Water activity of pesta granules

The pesta was incubated for 24 hours before the water activity measurements were made (**Table 4.5**).

Table 4.5 The water activity (A_w) and temperature of the pesta granules.

BRG*gfp*-15		Untreated	
A_w	Temp (ºC)	A_w	Temp (ºC)
0.214	20.67	0.583	20.07

The water activity of the treated pesta was 0.214. The untreated control was 0.583.

4.3.2.3 Bacterial enumeration of pesta

The number of viable cells of BRG*gfp*-15 in the pesta was enumerated following 72 hours of storage at 4ºC. The untreated control was enumerated in order to ensure no background contamination of the pesta by its dry ingredients or during its preparation.

There were no bacteria found in the untreated control (lowest dilution was 10^{-4}). Pesta containing BRG*gfp*-15 had 5.22 x 10^7 cfu/g.

4.3.2.4 Enumeration of BRG*gfp*-15 colonizing specific green foxtail root sections

Washings from the specific sections of the roots of green foxtail seedlings revealed the overall number of viable cells/ gram root material for BRG*gfp*-15 when formulated into pesta granules (**Table 4.6**). The root was divided lengthwise into equal parts. The first section was the proximal1/3 section along with the seed. The next was the middle 1/3 section and the last was the distal 1/3 section. The results are presented as both colony-forming units per gram dry weight of the root and its log value. Microbial counts were performed on untreated pesta (M9 molasses media with no bacteria) and no bacteria were found from washings of the root.

Table 4.6. BRG*gfp*-15 colonization on the three lengthwise sections of green foxtail roots following 14 days of incubation in growth pouches inoculated with pesta.

Section	Experiment	cfu/ml	cfu/g	Log10 cfu/g	Letter Grouping
Proximal	1	3.86E+06	4.14E+09	9.62	
	2	1.82E+06	1.95E+09	9.29	
	3	9.55E+05	1.07E+09	9.03	
	Mean	**2.21E+06**	**2.39E+09**	**9.31**	**A**
Middle	1	3.20E+03	5.61E+06	6.75	
	2	6.03E+04	1.09E+08	8.04	
	3	5.11E+04	1.14E+08	8.06	
	Mean	**3.82E+04**	**7.62E+07**	**7.61**	**B**
Distal	1	2.13E+03	4.89E+06	6.69	
	2	1.78E+04	5.21E+07	7.72	
	3	1.87E+04	4.20E+07	7.62	
	Mean	**1.29E+04**	**3.30E+07**	**7.34**	**B**

*Means with the same letter are not significantly different, as determined by analysis with SNK. Significance level was 0.05%.

The proximal section of the root had $\log_{10}9.31$ viable cells/g dry root weight, while the middle and distal sections had only $\log_{10}7.61$ and $\log_{10}7.34$, respectively. Statistical analysis revealed a significant difference between the proximal 1/3 section and seed as compared to the middle and distal sections. BRG*gfp*-15 showed a preference for colonizing the proximal 1/3 section and seed; however, BRG*gfp*-15 cells were also found on the middle and distal sections of the root (**Figure 4.10**). There was no significant difference between the number of bacterial cells colonizing the middle and distal sections of the root.

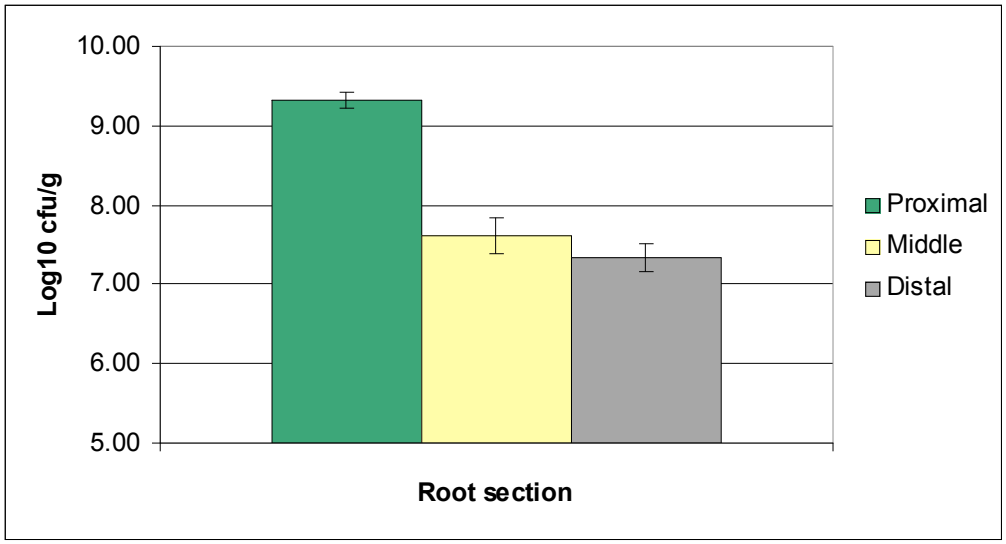


Figure 4.10. BRG*gfp*-15 colonization on the three lengthwise sections of green foxtail roots following 14 days of incubation in growth pouches.
Error bars represent the standard error of the mean.

4.3.2.5 Observation of BRG*gfp*-15 colonized on green foxtail roots under the confocal and epifluorescent microscopes

A total of 27 seedlings were observed under the epifluorescent microscopes (see **Appendix H**). All three sections of the root were colonized, as was observed with the liquid inoculum, however there was a definite preference for the proximal1/3 section + seed, which was typically more heavily colonized than the middle and distal sections. This was supported by the data obtained from spiral plating. The pesta granules contained fluorescent residue along the root mainly in the upper section (**Figure 4.11a**). The purple colour (note white arrows) observed was most likely autofluoresence from the organic peat in the pesta. The bacterial cells were often found still encased in the pesta granules, but also appeared to migrate from the pesta granule onto the root (**Figure 4.11b)**. Sometimes individual cells could be seen within the pesta granules (**Figure 4.11c**). It was also commonly observed that a large number of bacterial cells had migrated from the pesta granules and had colonized along the root (**Figure 4.11d).**

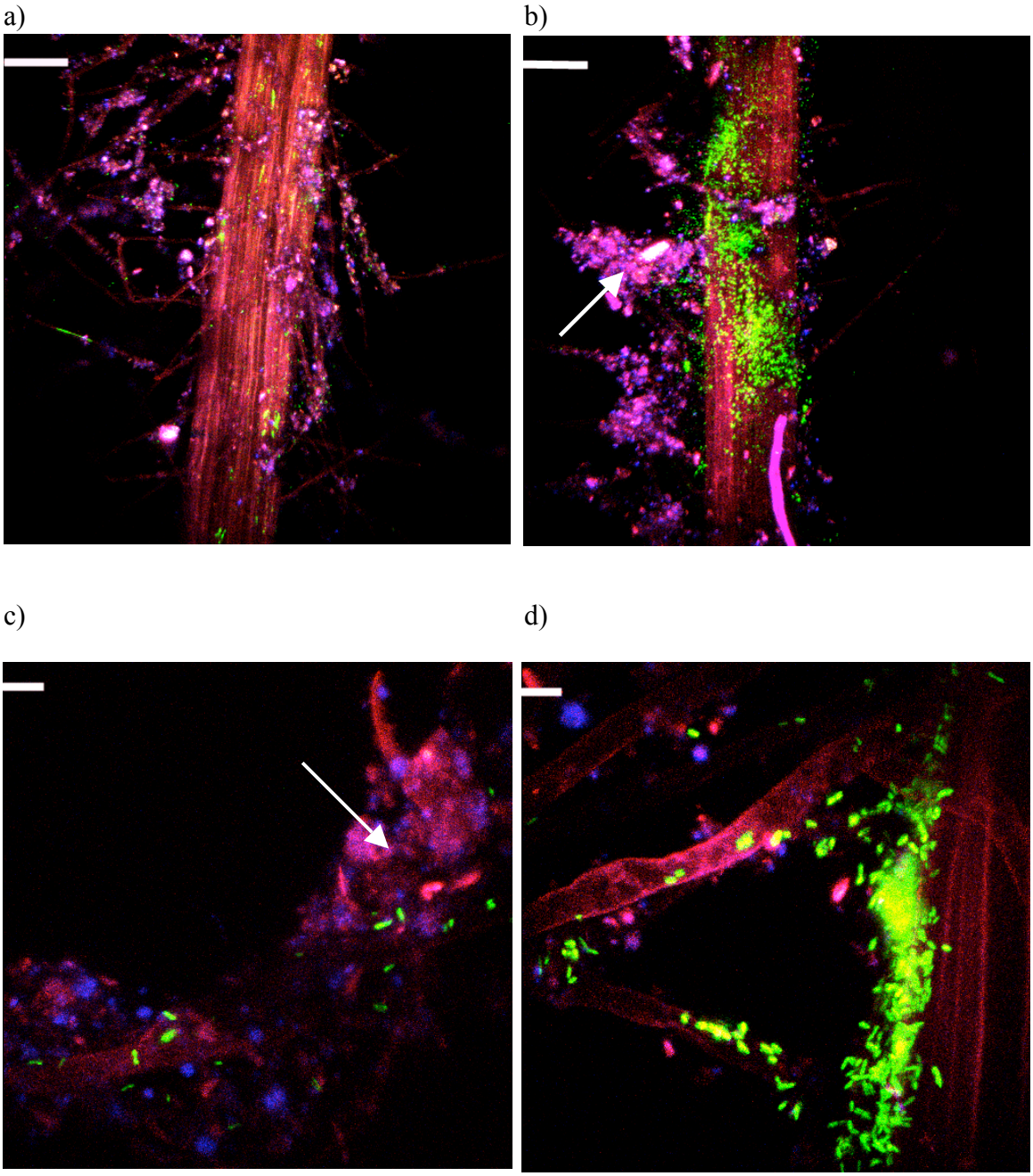
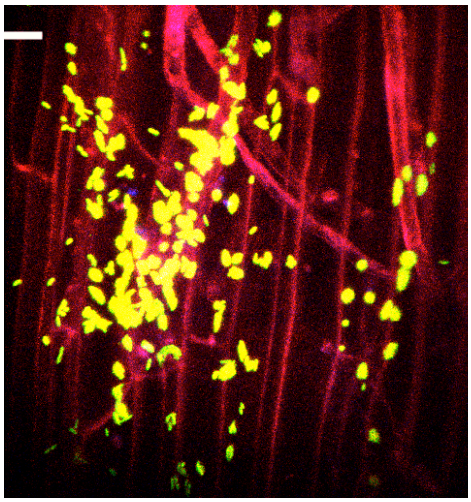


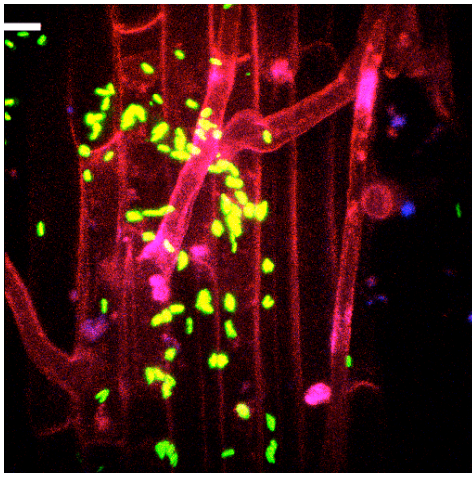
Figure 4.11. BRG*gfp*-15 on the roots of green foxtail seedlings observed using confocal microscopy.
a) and b) 10x Nikon water immersible NA 0.3; c) and d) 63x Zeiss water immersible NA 0.9. *White scale bars represent 100µm for a) and b) and 10µm for c) and d); note white arrows indicating the location of (purple) autofluorescence of peat located in the pesta granules.

As with the liquid inoculum, cells of BRG*gfp*-15 were found as both microcolonies and spread out as individual cells (**Table 4.12a** and **Table 4.12b**) and the root hairs were also heavily colonized (**Table 4.12c**). Bacterial cells were not in a clear pattern. In general the colonization from the pesta granules resulted in more concentrated areas of colonization the cells and were not as evenly dispersed as with the liquid inoculum. Furthermore, cells colonizing the edges of epidermal cells were occasionally observed. Sometimes it was observed that cells were colonizing along the edges of epidermal cells. Light colonization of the distal 1/3 section was often seen. The distal section of the root often had the lightest colonization, but often there was an increase at and near the root tip, as seen with the liquid inoculum.

a)



b)



c)

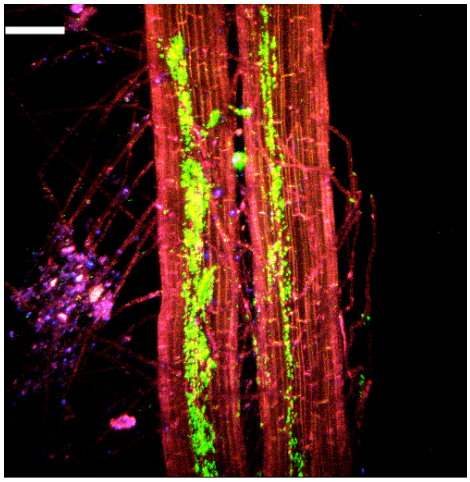


Figure 4.12. BRG*gfp*-15 on the roots of green foxtail seedlings observed using confocal microscopy.
a) and b) 63x Zeiss water immersible NA 0.9; c) 10x Nikon water immersible NA 0.3. *White scale bars represent 10µm for a) and b) and 100µm for c). (Note: BRG*gfp*-15 cells appear yellow in some areas due to excitation and emission wavelength used)

4.4 Effect of soil moisture, temperatures, and type on BRG*gfp*-15 proliferation and survival in soil using the thermogradient plate.

4.4.1 Determination of soil characteristics

The soils collected from the Saskatoon and Scott Research farms were analyzed for soil pH, texture, and nitrogen, phosphorus, potassium, and sulfur (**Table 4.7**).

Table 4.7. Summary of soil characteristics.

Parameter	Location: Saskatoon	Location: Scott
pH	7.9	4.7
Texture	**Clay**	**Clay loam**
Available N	122 mg/kg	72 mg/kg
Available S	25 mg/kg	14 mg/kg
Available P	18 mg/kg	54 mg/kg
Available K	520 mg/kg	540 mg/kg

The Saskatoon soil was a clay soil, whereas the Scott soil was a clay loam. The Saskatoon soil had a pH of 7.9 and the Scott soil had a pH of 4.7. The amount of available N was 122 mg/kg and 72 mg/kg for Saskatoon and Scott, respectively. At the Saskatoon site, the amount of available sulphur was 25 mg/kg, available phosphorus was 18 mg/kg, and potassium was 520 mg/kg. At the Scott site the amount of available sulphur was 14 mg/kg, available phosphorus was 54 mg/kg, and potassium was 540 mg/kg. As already mentioned, the Saskatoon soil was a clay soil and the Scott soil was a clay loam soil. Clay loam soil is composed of approximately 30-40% clay, 60 to 70% silt, and 35 to 60% sand. Clay soil is 55 to 100% clay, 0 to 40% silt, and 40-65% sand (**Figure 4.13**).

*adapted from www.soilsensor.com/soiltypes.aspx

Figure 4.13. The soil texture triangle.

4.4.2 Determination of soil moisture holding capacity

Moisture holding capacity was determined for the Saskatoon and Scott soils. In the case of the Saskatoon soil, 64 ml of sterile water was added before the first drop was observed. Therefore, the soil moisture holding capacity was 64%. For 25% soil moisture holding capacity, 25 percent of 64 ml of water was added, and similarly 50% and 75% of 64 ml. In the case of the Scott soil moisture holding capacity was 48%. These findings were confirmed with a percent moisture test. The soil plus the appropriate volume of water was weighed then placed in a dry oven at 101ºC for 24 hours. The soil was weighed again and the percent moisture calculated as follows: weight after drying / weight before drying = x. 1-x *100 = % moisture.

4.4.3 Effect of soil moisture, temperature, and type on BRG*gfp*-15 proliferation and survival in soil using the thermogradient plate.

Pesta granules containing BRG*gfp*-15 were enumerated prior to setting up the thermogradient plate experiment in order to obtain day 0 baseline counts per gram of soil. The mean Log_{10} cfu/g number of bacteria of each of the 18 treatments at day 0, day 14, day 28, and day 42 were obtained (**Table 4.8**). The initial concentration of 6.28 Log_{10} cfu/g was obtained one week before pesta was placed in thermogradient plate. Data from experiments 1 and 2 has been combined.

Table 4.8 Enumeration of BRG*gfp*-15 in pesta granules incubated in the thermogradient plate.

	Soil moisture:		0 days	14 days	28 days	42 days
Temperature ºC	% Field capacity	Soil type	Log10 cfu/g			
5 to 15	25%	Saskatoon	6.28	10.51	10.36	10.26
5 to 15	50%	Saskatoon	6.28	10.44	10.46	10.02
5 to 15	75%	Saskatoon	6.28	10.42	10.49	9.71
5 to 15	25%	Scott	6.28	9.79	9.49	9.51
5 to 15	50%	Scott	6.28	9.51	9.34	9.45
5 to 15	75%	Scott	6.28	9.24	8.91	8.49
15 to 25	25%	Saskatoon	6.28	10.15	10.52	9.55
15 to 25	50%	Saskatoon	6.28	9.95	9.70	9.59
15 to 25	75%	Saskatoon	6.28	8.64	7.47	6.54
15 to 25	25%	Scott	6.28	9.41	9.40	9.53
15 to 25	50%	Scott	6.28	9.38	9.53	9.38
15 to 25	75%	Scott	6.28	8.47	7.65	6.50
25 to 35	25%	Saskatoon	6.28	8.50	8.62	8.46
25 to 35	50%	Saskatoon	6.28	8.75	7.61	6.37
25 to 35	75%	Saskatoon	6.28	7.70	6.49	5.69
25 to 35	25%	Scott	6.28	8.51	8.45	7.44
25 to 35	50%	Scott	6.28	7.37	6.43	6.27
25 to 35	75%	Scott	6.28	7.44	6.30	5.66

The number of viable BRG*gfp*-15 cells/g over a 42-day period in the thermogradient plate under the various conditions of the 18 treatments is shown in **Figure 4.14, Figure 4.15,** and **Figure 4.16**. The data is divided according to the 3

temperature regimes in order to simplify the presentation of data and make the information more visible.

The following results are for the temperature regime of 5-15°C (**Figure 4.14**). At day 14, the number of viable cells/g in the Saskatoon soil was $\log_{10}10.51$, $\log_{10}10.44$, and $\log_{10}10.42$ for 25, 50, and 75% moisture, respectively. In the Scott soil the number of viable cells/g were $\log_{10}9.79$, $\log_{10}9.51$, and $\log_{10}9.24$ for 25, 50, and 75% soil moisture holding capacity, respectively. At day 28, the number of viable cells/g in the Saskatoon soil was $\log_{10}10.36$, $\log_{10}$ 10.46, and $\log_{10}10.49$ for 25, 50, and 75% moisture holding capacity, respectively. In the Scott soil the number of viable cells/g were $\log_{10}9.49$, $\log_{10}9.34$, and $\log_{10}8.91$, respectively. At day 42 in the Saskatoon soil the number of viable cells/g was $\log_{10}10.26$ for 25% moisture holding capacity, $\log_{10}10.02$ for 50% moisture holding capacity, and $\log_{10}9.71$ for 75% moisture holding capacity. In the Scott soil the number of viable cells/g were $\log_{10}9.51$, $\log_{10}9.45$, and $\log_{10}8.49$, for 25, 50, and 75% moisture holding capacity, respectively.

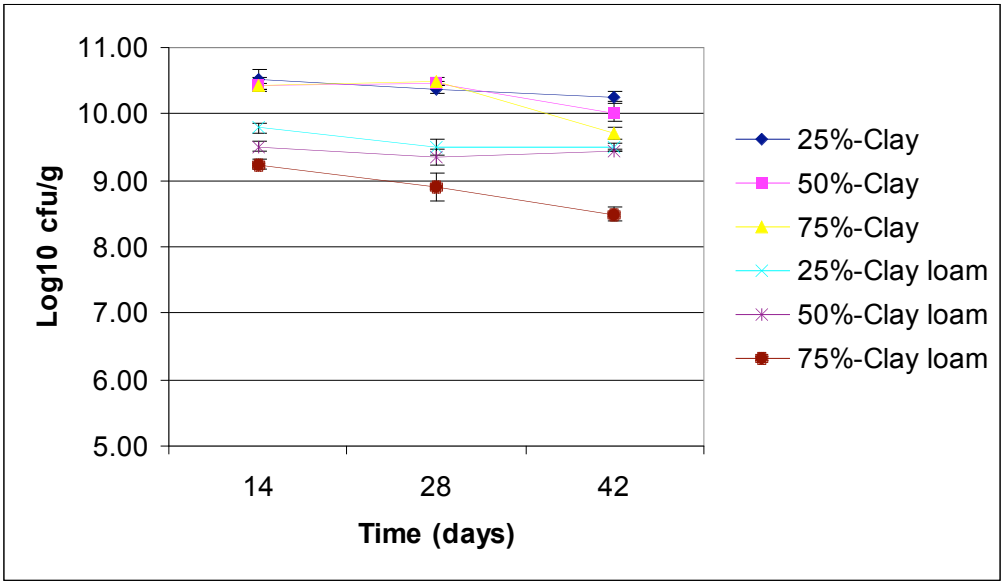


Figure 4.14. Enumeration of BRG*gfp*-15 in pesta granules incubated in the thermogradient plate in a temperature cycle of 5-15ºC over a 42 day period. Error bars represent the standard error of the mean

The following results are for the temperature regime of 15-25°C (**Figure 4.15).** At day 14, the number of viable cells/g in the Saskatoon soil was $\log_{10}10.15$ and $\log_{10}9.95$, for 25 and 50% moisture holding capacity, respectively. The number of viable cells/g for 75% moisture holding capacity was $\log_{10}8.64$. In the Scott soil the number of viable cells/g were $\log_{10}9.51$, $\log_{10}9.38$, and $\log_{10}8.47$, for 25, 50, and 75% moisture holding capacity, respectively. At day 28, the number of viable cells/g in the Saskatoon soil was $\log_{10}10.52$, $\log_{10}9.70$, and $\log_{10}7.47$, for 25, 50, and 75% moisture holding capacity, respectively. In the Scott soil the number of viable cells/g were $\log_{10}9.40$, $\log_{10}9.53$, and $\log_{10}7.65$, for 25, 50, and 75% moisture holding capacity. At day 42 in the Saskatoon soil the number of viable cells/g was $\log_{10}9.55$ for 25%, $\log_{10}9.59$ for 50%, and $\log_{10}6.54$ for

75% moisture holding capacity. In the Scott soil the number of viable cells/g was $\log_{10}9.53$ for 25%, $\log_{10}9.38$ for 50%, and $\log_{10}6.50$ for 75% moisture holding capacity.

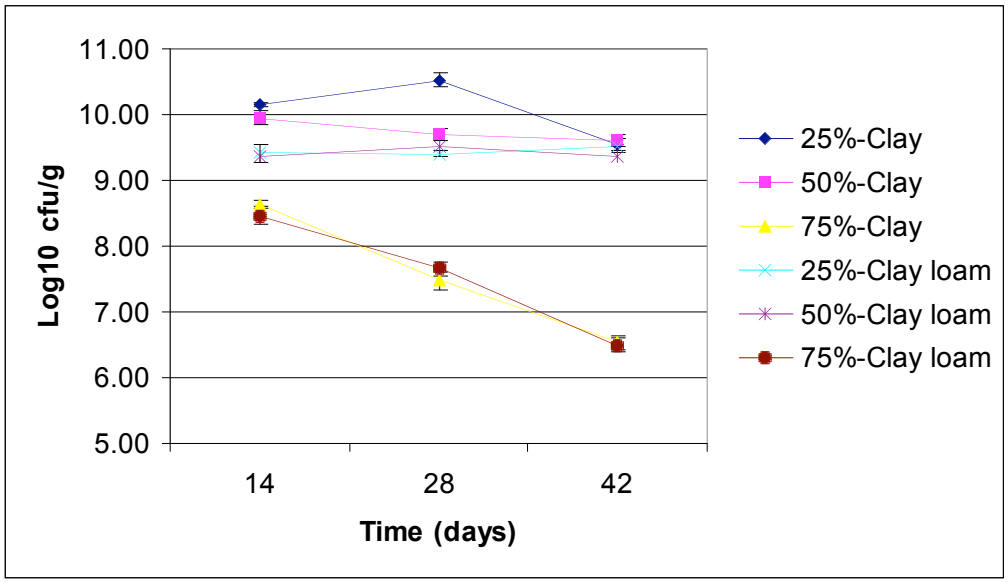


Figure 4.15. Enumeration of BRG*gfp*-15 in pesta granules incubated in the thermogradient plate in a temperature cycle of 15-25ºC over a 42 day period. Error bars represent the standard error of the mean

The following results are for the temperature regime of 25-35°C (**Figure 4.16**). At day 14 in the Saskatoon soil the number of viable cell/g was $\log_{10}8.50$ for 25% moisture holding capacity. For 50 and 75% moisture holding capacity the values were $\log_{10}8.75$ and $\log_{10}7.70$, respectively. In the Scott soil the number of viable cell/g was $\log_{10}8.51$ for 25% moisture holding capacity. For 50 and 75% moisture holding capacity the values were $\log_{10}7.37$ and $\log_{10}7.44$, respectively. At day 28 the number of cfu/g in the Saskatoon soil were $\log_{10}8.62$, $\log_{10}7.61$, and $\log_{10}6.49$ for 25, 50, and 75% moisture holding capacity, respectively and in the Scott soil were $\log_{10}8.45$, $\log_{10}6.43$, and $\log_{10}6.30$ for 25, 50, and 75% moisture holding capacity, respectively. At day 42 the number of viable cells/g was $\log_{10}8.46$ for 25%, $\log_{10}6.37$ for 50%, and $\log_{10}5.69$ for

75% moisture holding capacity for the Saskatoon soil. The number of viable cells/g was $\log_{10}7.44$ for 25%, $\log_{10}6.27$ for 50%, and $\log_{10}5.66$ for 75% moisture holding capacity for the Scott soil.

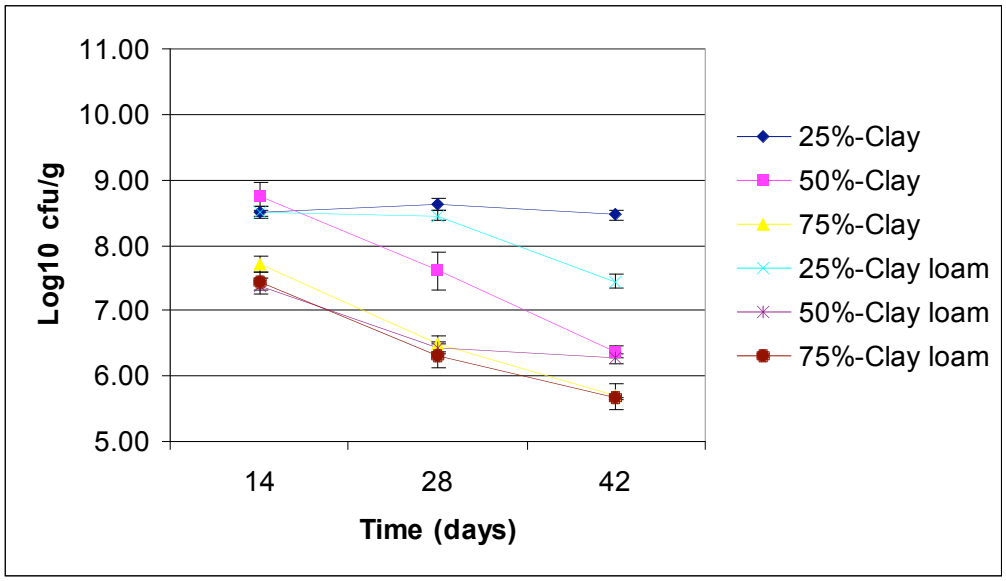


Figure 4.16. Enumeration of BRG*gfp*-15 in pesta granules incubated in the thermogradient plate in a temperature cycle of 25-35ºC over a 42 day period. Error bars represent the standard error of the mean.

The mean number of viable BRG*gfp*-15 cells/g of for each of the three temperature regimes used in the thermogradient plate: 5-15ºC, 15-25ºC, and 25-35ºC were tabulated (**Table 4.9**). There was a significant difference between the three temperature regimes. The highest mean number of viable BRG*gfp*-15 cells/g was seen with the 5-15ºC followed by the 15-25ºC, and the lowest number of viable BRG*gfp*-15 cells/g was seen with the 25-35ºC-temperature regime, $\log_{10}9.80$, $\log_{10}8.96$, and $\log_{10}7.33$, respectively.

Table 4.9. The mean number of viable BRG*gfp*-15 cells per gram of pesta incubated under the 3 temperature regimes after 42 days of incubation.

Temperature	ºC	Mean (log10 cfu/g)
1	5-15	9.80 A
2	15-25	8.96 B
3	25-35	7.33 C

*Means with the same letter are not significantly different as determined with SNK (p= 0.05). Note: Interactions between temperature and other factors was significant (see **Table 4.13**).

The mean number of viable BRG*gfp*-15 cells/g for each of the three soil moisture levels used in the thermogradient plate: 25, 50, and 75% of soil moisture holding capacity were tabulated (**Table 4.10**). There was a significant difference between all three moisture levels with the highest mean number of viable cells/g in the 25% soil moisture holding capacity followed by 50% and then 75%, $\log_{10}9.36$, $\log_{10}8.86$, and $\log_{10}7.87$, respectively.

Table 4.10. The mean number of viable BRG*gfp*-15 cells per gram of pesta incubated under the 3 soil moisture regimes after 42 days of incubation.

Soil moisture	% Field capacity	Mean (log10 cfu/g)
1	25	9.36 A
2	50	8.86 B
3	75	7.87 C

*Means with the same letter are not significantly different as determined with SNK (p = 0.05). Note: Interactions between moisture and other factors was significant (see **Table 4.13**).

The mean number of viable BRG*gfp*-15 cells/g for each of the two soil types was tabulated (**Table 4.11**). There was a significant difference between the two soils; the highest mean number of viable cells/g was seen in the Saskatoon soil (clay), which was $\log_{10}9.00$, compared to $\log_{10}8.40$ in the Scott soil (clay loam).

Table 4.11. The mean number of viable BRG*gfp*-15 cells per gram of pesta incubated under the 2 soil types after 42 days of incubation.

Soil type		Mean (log10 cfu/g)
1	Saskatoon (clay)	9.00 A
2	Scott (clay loam)	8.40 B

*Means with the same letter are not significantly different as determined with SNK (p = 0.05). Note: Interactions between soil type and other factors was significant (see **Table 4.13**).

The mean number of viable BRG*gfp*-15 cells/g of the three sampling times: 14, 28, and 42 days were tabulated (**Table 4.12**). There was a significant difference between all three sampling times. The highest mean number of viable cells/g was seen at 14 days followed by 28 days, then by 42 days, $\log_{10}9.12$, $\log_{10}8.74$, and $\log_{10}8.24$, respectively.

Table 4.12. The mean number of viable BRG*gfp*-15 cells per gram of pesta at the 3 sampling times.

Time	Days	Mean (log10 cfu/g)
1	14	9.12 A
2	28	8.74 B
3	42	8.24 C

*Means with the same letter are not significantly different as determined with SNK (p = 0.05). Note: Interactions between time and other factors was significant (see **Table 4.13**).

The significance of the effect on the mean number of viable cells/g of the various factors individually: time, temperature, soil moisture, and soil type, as well as the significance of the interactions between the various factors, was determined (**Table 4.13**).

Table 4.13. The significance of the effect of the various factors, as well as the significance of the interactions between the various factors on the mean number of viable cells of BRG*gfp*-15 shown as P-values.

Factor	P value
Experiment	0.1046
Time	<. 0001
Temperature	<. 0001
Moisture	<. 0001
Type	<. 0001
Time*temperature	< .0001
Time*moisture	< .0001
Time*type	0.0426
Temp*moisture	<. 0001
Temp*type	<. 0001
Moisture*type	0.1807
Time*temp*moisture	<. 0001
Time*temp*type	0.6256
Time*moisture*type	0.0615
Time*temp*moisture*type	<. 0001

* Factors having a p value of less than 0.05 are significant ($\alpha = 0.05\%$)

The statistical analysis shows the effect of the different sampling times, temperatures, soil moistures, and soil types on the mean number of viable cells/g are all very significant ($p < .001$). The effect of the interaction between temperature and soil moisture on the mean number of viable cells/g was also very significant. The effect of the interaction between temperature and soil type was significant. The effect of the interaction between soil moisture and soil type was not significant and the effect of the interaction between temperature, moisture, and type was almost significant. It was not significant at the set level of 0.05%, but if the significance level were set at 0.10% it would be significant.

5 Discussion

Rhizobacteria such as *Pseudomonas fluorescens* have great potential for biocontrol because of their ability to selectively reduce the growth of weed species but not interfere with the growth of the crop plant itself (Owen and Zdor, 2001). The parental strain *P. fluorescens* BRG100 has been found to be highly suppressive to the growth of green foxtail seedlings, resulting in up to 83% weed control (Daigle *et al.,* 2002). In the present study, two main hypotheses were tested. The first is that BRG*gfp*-15 would preferentially colonize certain areas of green foxtail roots. The second is that survival of BRG*gfp*-15 would be affected by different environmental conditions such as temperature and moisture in soil.

5.1 Selection of *gfp*+ mutant strain

The green fluorescence protein gene was introduced into *P. fluorescens* BRG100 from *E. coli* S17-λ1 carrying the suicide vector pAG408. Out of a total of 100 colonies per plate, 12-18 *gfp*+ colonies were found on each plate. This result is similar to past studies in which about 5% of transformants exhibited a bright green fluorescence (Suarez *et al*., 1997). Sixteen gfp+ isolates were subjected to a variety of assays to determine which of the isolates was most similar to the wild-type, BRG100. These assays included comparisons of morphology, biocontrol efficacy, carbon utilization, growth rate determination, and root colonization ability.

All sixteen isolates were found to have identical morphological characteristics to that of the wild-type. The ability of the isolates to suppress green foxtail root growth was tested against the wild-type. However the biocontrol efficacy of only seven of the sixteen

isolates was determined to be sufficient to warrant further examination. These isolates included BRG*gfp*-2, BRG*gfp*-5, BRG*gfp*-6, BRG*gfp*-13, BRG*gfp*-14, BRG*gfp*-15, and BRG*gfp*-16. This suggests that one or more genes important in the suppression of green foxtail root growth were interrupted by the insertion of the *gfp* gene. BRG*gfp*-7 lost the ability to cause root suppression; therefore one or more genes absolutely essential for biological control efficacy were interrupted by Tn5 transposition.

There are a number of factors involved in successful biocontrol, which may have been altered during the transformation with gfp. One possibility is that genes involved in the movement and/or colonization of the bacteria were interrupted. Motility is known to be a major trait needed for root colonization of tomato seedlings. Weert *et al*. (2002) determined that flagella-driven chemotaxis towards root exudates was the main reason behind this. They constructed mutants deficient in flagella-driven chemotaxis and found that mutants that retained their motility but were impaired in chemotaxis had significantly decreased ability for colonization and survival on tomato roots (Weert *et al.,* 2002). Additionally, Van der Bij *et al.* (1996) determined that flagella and the O-antigenic side chain of lipopolysacharides are vital to the colonization process.

Another possibility is that genes involved in the production or transport across membranes of various compounds known to contribute to weed suppression by rhizobacteria were interrupted. The production of secondary metabolites and phytotoxins are known to cause the characteristic root discolouration and reduced root length of weed plants by rhizobacteria, many of which also affect lipid synthesis and membrane integrity (Boyetchko *et al.,* 2002).

Pseudomonas fluorescens BRG100 is known to produce two cyclic lipodepsipeptides, pseudophomins A and B. Pseudophomin B is thought to be mainly responsible for the suppression of diseases caused by the fungi *Phoma lingam*/ *Leptosphaeria maculans* and *Sclerotinia sclerotiorum,* while pseudophomin A is more suppressive of green foxtail root germination than pseudophomin B (Pedras *et al.,* 2003). Pseudophomin A is a diastereomer of massetolide A and pseudophomin B is a diastereomer of massetolide C. Quail *et al*. (2002) established the chemical structure of these metabolites through a combination of spectroscopic techniques, chemical derivatization and degradation, and X-ray crystallography. The mode of action of some lipodepsipeptides is thought to involve the formation of ion channels that are permeable to divalent cations. This results in cell necrosis due to passive ion fluxes across the membrane (Pedras *et al.,* 2003). Since pseudophomin A is known to be an important factor in the suppression of green foxtail root germination, it is possible that production of this metabolite may have been compromised by the insertion of the *gfp* gene in those *gfp*+ isolates with reduced biocontrol efficacy in the present study.

Pseudomonas fluorescens D7 is known to produce the complex phytotoxin D7 among others, when colonizing the roots of downy brome, a weed affecting both winter wheat and fall rye (Gurusiddaiah *et al.,* 1994). Other examples of phytotoxins produced by pseudomonads are phaseoltoxin by *P. syringae* pv. *phaseolicola*, tabtoxin produced by *P. syringae* pv. *tabaci*, and syringomycin produced by *P. syringae* pv. *syringae* (Tranel *et al.,* 1993).

The mode of action by which rhizobacteria suppress the root growth of weed plants is not completely understood. However some secondary metabolites, including the

production of siderophores, hydrogen cyanide, or extracellular polysaccharides or the overproduction of indoleacetic acid, are thought to play a role (Kremer *et al.,* 2006). Rhizobacteria can suppress weed growth through overproduction of cyanide in that large amounts of hydrogen cyanide slow down root respiration, indirectly impairing nutrient acquisition (Shippers *et al.,* 1987). Cyanide inhibits cytochrome oxidase, which in turn stunts ATP production in plant cells (Owen and Zdor, 2001).

Kremer and Souissi (2001) reported that the concentration of HCN was directly related to specific rhizobacteria isolates' ability to suppress weed growth. Furthermore, HCN was found to inhibit velvetleaf growth, a leading weed in corn fields, but did not inhibit maize seedling growth. Although the corn rhizosphere had higher levels of HCN compared to that of velvetleaf, the growth of corn seedlings were not reduced. It seems that inhibition by HCN is species specific. Corn roots may exhibit some type of cyanide resistance (Owen and Zdor *et al.,* 2001).

Extracellular polysaccharides help bacteria in the colonization process and also cause wilting of the weed plant by interfering with water movement in xylem vessels (Fett *et al.,* 1989). Polysaccharides are thought to be required for biocontrol activity of *P. fluorescens* D7 against downy brome serving as a physical matrix that protects and stabilizes the active D7 toxin. Lipopolysaccarides are believed to have a function in the toxicity of several other *P. fluorescens* strains, as well (Gurusiddaiah *et al*., 1994).

Rhizobacteria have also been found to reduce weed growth through the production of high amounts of indole compounds. Sarwar and Kremer (1995) reported enhanced growth inhibition of several weed species by a rhizobacterium, which produced several indole compounds, IAA at a concentration of 102 $\mu g\ L^{-1}$, indole-3-aldehyde at a

concentration of 0.4µg L^{-1}, and indole-3-lactic acid at a concentration of 7.6 µg L^{-1}. These three compounds are known to inhibit plant growth. Therefore, one possibility why many of the *P. fluorescens gfp*+ isolates had reduced biocontrol efficacy may have been that genes involved in the production of any of these secondary metabolites or phytotoxins were interrupted.

Carbon utilization profiles of the *P. fluorescens gfp*+ isolates were compared to that of the wild-type. Out of the seven isolates with comparable biocontrol efficacy of green foxtail to that of the wild-type BRG*gfp*-2, 5, 6, 13, 14, 15, and 16, only four had similar carbon utilization patterns to the wild-type. These isolates had only one to three differences in carbon utilization to that of the wild-type. Some other isolates had as many as 13 or 14 differences in carbon utilization compared to that of the wild-type. One isolate had 22 differences, suggesting several genes needed to utilize these carbon sources may have been interrupted while acquiring the *gfp* gene.

It is vital that the isolate chosen have a highly similar carbon utilization profile to the wild-type because root exudation is the major plant-derived factor responsible for initiating root colonization. Plants secrete about 10-44% of their photosynthates as root exudates (Rudrappa *et al.,* 2008). Therefore changes in the ability of a mutant to utilize these sources can greatly affect biocontrol efficacy.

Growth in liquid media by *P. fluorescens* BRG*gfp*-5, 14, 15, and 16 and the wild-type were compared. The doubling times for BRG*gfp*-5, 14, 15, and 16 were 10.8, 9.4, 8.1 and 11.4 hours, respectively. The doubling time for the wild-type was 8.7 hours. Therefore, BRG*gfp*-15 has the most similar doubling time to the wild-type. BRG*gfp*-16 grew more slowly in liquid media than the wild-type. As determined by optical density

readings, BRG*gfp*-16 reached log phase 21 hours later than BRG100. At 26 hours the concentration of BRG100 was much higher than the concentration of BRG*gfp*-16. Clearly genes vital to the growth of BRG*gfp*-16 were interrupted by the insertion of the *gfp* gene.

BRG*gfp*-14 and 15 both had similar growth kinetics to the wild-type although BRG*gfp*-14 and 15 lagged a few hours behind with the beginning of their log phase. As previously stated, the doubling times for BRG*gfp*-14 and BRG*gfp*-15 were 9.4 and 8.1 hours, respectively, both similar to the wild-type which was 8.7hours. However, BRG*gfp*-15 was selected over BRG*gfp*-14 as the *gfp*+ isolate for all further experiments in part because it emitted a much stronger fluorescence than BRG*gfp*-14. BRG*gfp*-16 also emitted a very strong fluorescence but the growth in liquid media had clearly been impaired. The liquid inoculum must reach a sufficient concentration after 48 hours of incubation for all subsequent experiments therefore BRG*gfp*-16 was not suitable. In addition to emitting a strong fluorescence, BRG*gfp*-15 was selected for all subsequent experiments because it had similar biocontrol efficacy, a similar carbon utilization profile, and similar growth in liquid media to the wild-type.

5.2 Root colonization of green foxtail seedlings by *Pseudomonas fluorescens* BRG*gfp*-15

BRG*gfp*-15 was tested against the wild-type, BRG100, for its ability to colonize the roots of green foxtail and wheat seedlings. There was no significant difference between the isolates as to the number of cells colonizing the roots of either plant; therefore BRG*gfp*-15 was considered suitable for the root colonization and thermogradient plate experiments.

Biocontrol agents are often limited in efficacy in field conditions because of unreliable root colonization. Therefore, identification of traits involved in successful root colonization can result in a more efficient product. This relies on a good understanding of the localization, organization, activity and viability of the active microbial agent along the root (Gamalero *et al.,* 2005). The root colonization pattern of BRG*gfp*-15 was examined by dilution plate count, confocal, and epifluorescent microscopy. When determined by dilution plate count the bacterium showed a preference for colonizing the proximal 1/3 section of the root, including the seed as compared to the middle and distal 1/3 section. BRG*gfp*-15 had significantly higher numbers of viable cells per gram of root dry weight in the proximal 1/3 section and the seed, both with liquid inoculum and when formulated into pesta granules, $\log_{10}9.06$ and $\log_{10}9.31$, respectively.

In past studies the primary location of root colonizing bacteria was the upper part of the main root and the number of bacteria progressively decreased down the roots (Beauchamp *et al.,* 1992; de Weger *et al.,* 1997). The proximal 1/3 section of the root is more mature than the distal section. One reason for a higher amount of colonization success on the proximal root section is that the emergence of lateral roots results in a high level of root exudate secretion encouraging microcolony formation (Rudrappa *et al.,* 2008).

Observation under the epifluorescent and confocal microscopes clearly showed that BRG*gfp*-15 was released from the pesta granules enabling it to colonize the green foxtail roots very well. It was also confirmed through epifluorescent microscopy that when applied in pesta granules, the bacterium had a preference for the proximal section. However, when applied as liquid inoculum it was not obvious during direct microscope

observation that BRG*gfp*-15 had a preference for the proximal section. Bacteria were commonly found heavily colonizing all three lengthwise sections. The difference may have not been obvious because the numbers of viable cells per gram of root were substantial for both the middle and distal 1/3 section ($\log_{10}$7.53 and $\log_{10}$7.01, respectively).

Observations under the epifluorescent and confocal microscopes enabled an intricate examination of the distribution along the root and added to the information gathered from enumeration studies from the three sections of root. Trends were found as to the specific regions that were heavily colonized and the patterns in which they were colonized. Root colonization by most bacterial strains is a dynamic process, influenced by the growth, development, and differentiation of the plant root (Gamalero *et al.,* 2005).

Chemicals secreted from the roots into the soil are broadly referred to as root exudates. Through the exudation of many of these compounds, roots can influence and regulate the microbial community in the immediately surrounding soil (Walker *et al.,* 2003). Region-specific variations in bacterial densities of the root were influenced by the patterns of root exudates concentration and composition in past studies (Rincon *et al*., 2005).

Nutrient availability is commonly accepted as one cause for non-uniform colonization of the rhizosphere. The areas of the root with the most exudation are the root tips, the area of and around the apical meristem, the region of elongation, and developing root hairs. However there are other factors known to affect root colonization patterns. Surface topography can play a large role in that surface irregularities at various locations

of the root provide protection from mechanical shearing and predators (Dundurand *et al*., 1997).

High numbers of BRG*gfp*-15 cells were found on the ventral portion of seeds. It is well documented that cracks in the seed coat are often highly colonized. This is because cracks and grooves on the seed form areas of protection from mechanical shearing and predators (Hoseney, 1986). In addition, when the seed first begins to germinate, cytoplasmic solutes leak from broken plant cells providing a high level of nutrients on the seed surface (Werker, 1997). BRG*gfp*-15 was also commonly found lining the edges of epidermal cells all along the root length either in microcolonies or in strings of closely associated cells. This is likely because of the protection provided by the groove between the cells, much the same as with cracks and grooves in the seed surface. Similar results have been reported in past studies (Unge and Jansson, 2001; Rincon *et al*., 2005).

In this study, BRG*gfp*-15 was rarely found at the very tip of the root. Although the root tip is identified as an area of high exudation, it has been found in past studies to be devoid of bacteria (Kragelund and Nybroe, 1996; Simons *et al*., 1996). This is likely due to the mechanical abrasion caused by the soil in which the root is growing or the high level of turnover of epidermal cells in the root elongation zone and apex, which may restrict effective colonization (Bowen and Rovira, 1976). The root tip has a maximum carbon turnover due to the continuous 'decapping' of the root cap (Rudrappa *et al.,* 2008). However, microcolonies were often found ~5-10 mm up from the root tip. This is most likely because this area does not experience the amount of mechanical shearing as the very tip, but still has a high level of root exudates (Kragelund and Nybroe, 1996; Simons *et al*., 1996).

5.3 Effect of soil moisture, temperatures, and type on BRG*gfp*-15 using the thermogradient plate.

It has been suggested that one of the fundamentals of weed biocontrol is that the microbial agent is exposed to favourable conditions in the surrounding environment. This can help optimize the ability of the pathogen to cause disease in the weed plant (Boyetchko, 2005). By distinguishing which soil conditions influence weed suppression, biocontrol efficacy can be improved (Ownley *et al.,* 2003). In the thermogradient plate experiment the survival of BRG*gfp*-15 at various soil temperature regimes, 5-15ºC, 15-25ºC, and 25-35°C, soil moistures, 25, 50, and 75% of moisture holding capacity, and soil textures, clay and clay loam, were examined. The survival and/or dispersion of BRG*gfp*-15 in pesta granules were significantly affected by these varying conditions. At the lowest temperature regime, 5-15°C, by day 14 the number of BRG*gfp*-15 viable cells/ gram of pesta had greatly increased with all 6 treatments. The number of BRG*gfp*-15 under all treatments was still much higher at the last sampling time than at day 0, indicating BRG*gfp*-15 can flourish under all 3 moisture levels as well as soil types with the 5-15°C temperature regime. There are a few significant trends that have been found with the 5-15°C regimes. The number of viable cells per gram of pesta for all three moistures for the Saskatoon soil were higher than that observed in the Scott soil. Furthermore, microbial counts were the highest at the lowest moisture, 25% moisture holding capacity in both types of soil followed by 50% moisture holding capacity and then 75% moisture holding capacity.

As with the 5-15ºC-temperature regime discussed above, at day 14 the counts for BRG*gfp*-15 cultivated under the 15-25°C temperature regime greatly increased with all 6 treatments. There was already a difference in BRG*gfp*-15 population density with respect to soil moisture level and soil type. There were some significant differences that are seen at the second temperature regime (15-25°C). The first was both the 25% and 50% soil moisture holding capacity for both soils had a higher number of cfu/g than the 75% moisture. This is different than what were observed with the lower temperature. There was less of a difference between the two types of soil. There was a significant interaction between the temperature, moisture, and texture effects.

For the last temperature regime examined, 25-35ºC, the number of viable cells/g was consistently lower than with the other two temperature regimes. As with the 15-25°C, the 25 % moisture holding capacity had the highest number of viable cells/g for both soil types, followed by the 50% moisture holding capacity, and then the 75% moisture holding capacity. However, there was less of a difference between the 50% and 75% moistures. When comparing the overall results from one temperature regime to the others, it was clear that 5-15°C overall has the highest number of cells per gram, followed by 15-25°C, and the lowest numbers were seen with the highest temperature regime, 25-35°C. There were significant interactions between almost all the factors. As determined statistically, the interactions between the following factors on the mean number of viable cells/g were very significant: time*temperature, time*moisture, temperature*moisture, temperature*type, time*temperature*moisture, and time*temperature*moisture*type. Time*type was also significant.

As mentioned previously, soil temperature had a significant effect on the survival of BRG*gfp*-15. The highest number of viable cells was found in the soil incubated at the lowest temperature (5-15ºC) and the lowest numbers were found at the highest temperature (25-35ºC). Similar results have been previously reported. Bowers and Parke (1993) found that soil temperature affected *P. fluorescens* colonization of pea plants. There was a decrease in population when the temperature was increased. The population was highest at 16˚C and decreased linearly with increasing temperature (Bowers and Parke, 1993).

The survival of bacterial cells may be enhanced at lower temperatures partially because rates of microbial activity and growth are more rapid at high temperatures and the cells can begin to use up all of the available nutrients. The optimum growth temperature of *P. fluorescens* was determined to be 25-30˚C and the greatest survival usually occurs at temperatures a bit below the optimum growth temperature (Bowers and Parke, 1993).

The results from the thermogradient plate experiment are further supported by the findings of a study by O'Callaghan *et al*. (2001). They reported that survival of *P. fluorescens* was greatest at 10ºC. The initial population was 8 x 10^5 cfu/g soil and after 110 days of incubation at 10ºC there was at least 1x 10^3 cfu/g. At 20ºC the population decline was much greater. Populations dropped below detectable levels after only 54 days (O'Callaghan *et al*., 2001).

Seong *et al*. (1991) reported that survival of *P. fluorescens* ANP15 was strongly affected by the ambient temperature. Biomass of *P. fluorescens* ANP15 was reduced as the soil temperature was increased. Survival was highest after 50 days at 4ºC and 20ºC,

and was reduced at temperatures of 28ºC and higher. Another reason that survival at elevated temperatures is reduced may be the thermal inactivation of proteins and cell structures (Seong *et al.,* 1991).

Population dynamics on the rhizoplane by *P. fluorescens* D7, a bacterium highly effective in the suppression of the weed plant downy brome, was investigated under different temperatures. Populations were two to three log units higher at a temperature regime of 10/10ºC than at 18/13ºC and 25/15ºC. Weed growth suppression was also highest at the lowest temperature, which is beneficial because downy brome germinates in late autumn when temperatures are low (Johnson et al., 1993). Similarly, the enhanced survival of BRG*gfp*-15 at low temperatures is beneficial because pesta granules containing the wild-type BRG100 should be applied in early spring, as it is a pre-emergent biocontrol agent for green foxtail and wild oat.

Although survival of BRG*gfp*-15 at temperatures lower than 5°C was not investigated, it has been reported that colonization of soil and downy brome roots by *P. fluorescens* D7 declined late in the season (Kremer and Kennedy, 1996). Thus, annual application of *P. fluorescens* BRG100 for the control of green foxtail will likely require annual application in a field situation.

Survival of BRG*gfp*-15 was also significantly affected by soil moisture. The highest number of viable cells per gram was observed in the soil with the lowest moisture, 25% of moisture holding capacity. The number of viable cells of BRG*gfp*-15 were significantly lower at 50% and even lower at 75% of moisture holding capacity. In past studies, similar results have been found. Normander *et al.* (1999) observed a very significant affect of soil moisture on the survival of *P. fluorescens* DR54-BN14. At 160%

of moisture holding capacity, viability was greatly reduced, but at 10% moisture holding capacity numbers remained constant over an extended period of time (Normander *et al*., 1999).

Similarly, O'Callaghan *et al*. (2001) reported that survival was reduced at increasing soil moisture levels. The initial population of *P. fluorescens* was 8 x 10^5 cfu/g of soil. After 110 days populations declined to 1 x 10^4 cfu/g in soils with 23% moisture, 7 x 10^3 cfu/g with 30% moisture, and 1 x10^3 cfu/g with 13% moisture. All of the samples were incubated at 10ºC. In soils of the same three moisture levels but incubated at 15ºC, populations fell to between 3-5 x 10^2 cfu/g soil (O'Callaghan *et al*., 2001). This indicates a significant interaction between soil moisture and temperature as was observed in the thermogradient plate experiment with BRG*gfp*-15. In contrast with these findings *P. fluorescens* D7 was found to have reduced colonization ability and plant-suppressive activity at low soil water content. Migration of the organism was limited in dry soils due to a lack of percolating water.

For other soil-dwelling bacteria the opposite effect of soil moisture on survival has been observed. Cools *et al*. (2001) reported that survival of *Escherichia coli* and *Enterococcus* spp. was enhanced at increasing moisture levels. At 100% of moisture holding capacity survival was significantly higher than at 80% of moisture holding capacity and survival was the lowest at 60% of field capacity (Cools *et al.,* 2001). They proposed that lower soil moisture could cause drought stress for the bacteria, but this appears not to affect *P. fluorescens* BRG*gfp*-15 and other strains.

The soil type and texture are also known to influence survival of rhizobacteria. Various soil characteristics have been reported to affect colonization, metabolism,

survival, and biocontrol efficacy. Physical filtration and adsorptive forces affect the movement of bacteria through soil. A bacterium can move passively or actively through soil better if it can physically fit easily between soil particles, thus the pore size of the soil is important. If there is a sufficient amount of water in pores this will aid in the movement of bacteria through the soil (Bowers and Parke, 1993).

In a past study, biocontrol activity of *P. fluorescens* 2-79RN$_{10}$ was positively correlated with ammonium-nitrogen, percent sand, soil pH, sodium, and zinc. Biocontrol activity was negatively correlated with cation-exchange capacity (CEC), iron, percent clay, percent organic matter, percent silt, and total carbon and nitrogen. The most important soil factors out of these were determined to be ammonium-nitrogen, CEC, iron, percent silt, pH, and zinc (Ownley *et al.,* 2003).

In the thermogradient plate experiment clay soils collected from the AAFC Saskatoon research farm had a significantly higher number of viable BRG*gfp*-15 cells than clay loam soils collected from the Scott research farm. The Scott soil has a much lower percentage of clay and a higher percentage of silt than the Saskatoon soil, but relatively the same percentage of sand. The clay composition of the soil has been found to both support (Stutz *et al.,* 1989) and suppress root colonization and metabolic activity of *Pseudomonas* spp. (Ownley et al., 2003). Zdor *et al*. (2005) found that the suppression of green foxtail seedlings by *P. fluorescens* G2-11 was influenced by soil texture; the strongest suppression was seen in fine sandy loam. The low clay mineral content of the fine sandy loam soil may have favoured *P. fluorescens* G2-11 because of a reduction in interference of clay with phytotoxins (Zdor *et al.,* 2005).

As previously mentioned, biocontrol activity of *P. fluorescens* 2-79RN$_{10}$ was negatively correlated with percent clay. Clay has a large surface area with charges that attract ions and water. These surfaces provide areas for chemical reactions and nutrient exchange. It is possible that the negative influence of clay on biocontrol efficacy was due to a reduction in available nutrients that were adsorbed to the clay particles or was due to adsorption of anti-plant compounds (Ownley *et al.,* 2003).

In contrast to this, Wessendorf and Lingens (1989) found that fine textured soils favoured microorganisms. Fine textured soils had higher microbial populations than coarse textured soils. This is in agreement with the present study in which populations of BRG*gfp*-15 were higher in clay soil than in clay loam soil. Clay loam has a higher percentage of silt than clay soil and silt is composed of larger particles than clay. In addition to this soils rich in clay are thought to allow more microhabitats to be formed providing enough water and nutrients, as well as protection from predatory microorganisms (Wessendorf and Lingens, 1989).

The clay soil used in the present study had significantly higher numbers of viable BRG*gfp*-15 cells per gram of pesta than the clay loam soil. The pH of the clay soil was 7.9 and the pH of the clay loam was much lower, only 4.7. Many bacteria including *Pseudomonas* spp. prefer a neutral pH of 7; therefore the low pH of the clay loam may have been unfavourable to the survival of BRG*gfp*-15. As a general rule bacteria do not grow well at a pH below 5 (Van Bruggen *et al*. 2007). In one study, *P. fluorescens* 32 was incubated in two soils, both sandy with similar characteristics except for a different pH of 6.8 and 5.3. Populations of *P. fluorescens* 32 were higher in soils with higher pH. (Van Bruggen *et al.*, 2007).

In addition to survival in soil, biocontrol efficacy can be affected by soil pH. Owenly *et al.* (2003) investigated the biocontrol efficacy of *P. fluorescens* 2-79 on take-all disease of wheat in soil of varying pH from 5.17 to 8.53. Biocontrol efficacy was reduced as soil pH decreased (Ownley *et al.,* 2003). As mentioned previously the production of HCN is known to be one mechanism by which some rhizobacteria suppress weed growth. Owen and Zdor (2001) proposed that HCN production might be partially dependant on soil quality factors such as pH, inorganic phosphate, and ferric iron availability.

It is important to recognize that the reduction in the number of viable BRG*gfp*-15 cells per gram of pesta may be due in part to dissemination of the bacterial cells from the pesta granules into the surrounding soil. The survival and dissemination of various strains of *P. fluorescens* has been previously investigated. De Leij *et al.* (1995) found that *P. fluorescens* disseminated up to 2 m away from areas of inoculation and to depths over 45 cm in a field release experiment on wheat. In contrast to these findings vertical dissemination of a strain of *P. aureofaciens* was restricted to the rhizosphere of wheat. They postulated that differences in soil moisture and type might have contributed to these differences. Dissemination may be affected by soil type in that clay binds microbial cells more strongly than silt or sand (De Leij *et al*., 1995).

Natsch *et al*. (1994) observed vertical translocation of depths up to 60cm with *P. fluorescens* CHA0 when short but heavy rainfall was simulated (Natsch *et al.,* 1994). In the present study the lowest number of viable cells still found encased in pesta granules was highest at a moisture level of 75% of moisture holding capacity. It is possible that the higher soil moisture enhanced dissemination of BRG*gfp*-15 through the percolation of

water or diffusion. In one study, *P. fluorescens* D7 was found to have reduced colonization ability and plant-suppressive activity of downy brome at low soil water content. This was thought to be due to limited migration of the organism in dry soils because of a lack of percolating water and not to a reduction in population at low soil moisture (Johnson *et al.,* 1993). Therefore, higher soil moisture may enhance the dissemination of BRG*gfp*-15 from the pesta granules, encouraging root colonization of green foxtail.

6 Conclusions

6.1 Conclusions

A suitable *gfp*+ isolate of *P. fluorescens* BRG100 was produced by the transformation with the *gfp* gene and was named *P. fluorescens* BRG*gfp*-15. BRG*gfp*-15 was found to be similar to the wild-type BRG100 in morphology, weed biocontrol efficacy on green foxtail, carbon utilization, growth kinetics (doubling time) in liquid media, and root colonization of green foxtail and wheat seedlings.

The following conclusions were reached:

1. When applied to green foxtail seedlings as liquid inoculum and as pesta granules BRG*gfp*-15 had a preference for colonizing the proximal 1/3 root section and seed as compared to the middle and distal 1/3 root sections.

2. BRG*gfp*-15 also showed a preference for the root hairs, the ventral portion of the seed, and 5-10mm from the root tip.

3. BRG*gfp*-15 disseminated from the pesta granules and colonized green foxtail roots.

4. The number of viable cells of BRG*gfp*-15 per gram of pesta was significantly affected by soil temperature, moisture, and type. Lower soil temperatures of 5-15ºC had higher populations of BRG*gfp*-15 than 15-25ºC and populations were lowest at 25-35ºC. The lowest soil moisture, 25% moisture holding capacity, had the highest population of BRG*gfp*-15, followed by 50% and 75% moisture holding capacity. Soils collected from AAFC Saskatoon research farm, a clay soil, had higher populations of BRG*gfp*-15 than soils collected from AAFC Scott research farm, which was a clay loam soil.

5. The pesta formulation has proven very effective. Numbers of viable cells per gram of pesta were higher following 42 days of incubation than when initially inoculated into the soils. Survival of BRG*gfp*-15 in pesta granules was very high when exposed to a wide range of environmental conditions.

As previously stated, *P. fluorescens* BRG100 is in the process of being commercialized as a bioherbicide for the control of green foxtail. The conclusions made from this study will help in gaining registration approval for this biocontrol agent. The green fluorescent protein has been a very useful tool for monitoring and tracking BRG*gfp*-15 on green foxtail roots and in soil. *Pseudomonas fluorescens* BRG100 has been shown to be a successful colonizer of green foxtail and wheat roots and the pesta formulation has proven to be a success.

6.2 Further Research

Further research is needed in the following areas:

1. Examine the dissemination of BRG*gfp*-15 from pesta granules into the surrounding soil.

This will enable a better understanding as to whether the higher soil temperature and moisture decreases survival of BRG*gfp*-15 in pesta granules or enhances the dissemination of BRG*gfp*-15 into the surrounding soil. Therefore, another thermogradient plate experiment should be performed with the same soil temperatures, moistures, and types. Samples of soil could be taken at various distances from the row of pesta granules and the number of viable cells per gram of soil enumerated. This will enable a better understanding of what soil conditions affect dissemination of BRG*gfp*-15 from pesta granules in addition to survival.

2. Examination of the isolate BRG*gfp*-7 is needed.

BRG*gfp*-7 completely lost the ability to suppress the growth of green foxtail roots suggesting one or more genes essential for biocontrol efficacy had been interrupted. Further examination of this isolate was beyond the scope of this study; however, study of this isolate at the molecular level would provide valuable insight into understanding the mechanism by which BRG100 suppresses root growth of green foxtail.

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8 Appendices

8.1 Appendix A: Hoagland's solution

Hoagland's solution:

	[standard]	[10 %]	
Chemical stock solution		per L	per 10L
1M KH_2PO_4		1 ml	1 ml
1M KNO_3		5 ml	5 ml
1M $Ca(NO_3)_2$		5 ml	5 ml
1M $MgSO_4$		2 ml	2 ml
0.5 % Ferric tartrate		1 ml	1 ml
Microelement stock solution		1 ml *	1 ml *
Water		1000 ml	10,000 ml

* Microelement stock solution preparation (from stock solution concentrates)

Microelement concentrate**		per 100ml water
2.86 % Boric acid	H_3BO_3	10 ml
18.1 % Manganese chloride	$MnCl_2$	1 ml
2.20 % Zinc sulfate	$ZnSO_4{\sim}7H_2O$	1 ml
0.80 % Cupric sulfate	$CuSO_4{\sim}5H_2O$	1 ml
0.20 % Molybdic acid IV	$H_2MoO_4{\sim}H_2O$	1 ml

The following stock solution concentrates are prepared to improve accuracy and precision of the chemicals in solution and to facilitate rapid preparation. The concentrates prepared below are used to prepare the microelement stock solution required in the Hoagland's solution recipe

** Individual microelement stock solution concentrate preparation

Chemical	structure	[% w/v]	gr/L	[concentrate]	[%w/v]	g/200ml
Boric acid	H_3BO_3	0.286 %	2.86	10X	2.86 %	5.72 g
Manganese chloride	$MnCl_2{\sim}4H_2O$	0.181 %	1.81	100X	18.1 %	36.2 g
Zinc sulfate	$ZnSO_4{\sim}7H_2O$	0.022 %	0.22	100X	2.20 %	4.40 g
Cupric sulfate	$CuSO_4{\sim}5H_2O$	0.008 %	0.08	100X	0.80 %	1.60 g
Molybdic acid IV	$H_2MoO_4{\sim}H_2O$	0.002 %	0.02	100X	0.20 %	0.40 g

8.2 Appendix B: M9 Molasses Sucrose-Zinc Medium (M9MSZ)

Use: a zinc amended medium optimized for bacterial isolate BRG100 and found suitable for other Gram negative soil borne bacteria.

COMPONENTS	FORMULA	PER L.
Sodium phosphate (anhydrous)	Na_2HPO_4	6.0 g
Potassium phosphate (monobasic)	KH_2PO_4	3.0 g
Ammonium chloride	NH_4Cl	1.0 g
Sodium chloride	NaCl	2.0 g
Sucrose [0.2 % w/v final media]	$C_{12}H_{22}O_{11}$	2.0 g
Magnesium Sulfate, 7-hydrate	$MgSO_4$-$7H_2O$	1.23 g
Calcium chloride, di-hydrate	$CaCl_2$-$2H_2O$	0.015 g
Zinc sulfate, 7-hydrate [0.2 mM final media]	$ZnSO_4$-$7H_2O$	0.06 g
10 % w/v Molasses solution* [equivalent to 2 % v/v final media]		200 ml
*Add 20g molasses to a separate container; add 200ml water and mix to dissolve.		
Water	H_2O	800 ml
0.1 % Thiamine-HCL stock solution – add after sterilization.		5 ml

Stock solutions (store at 4 °C):
Thiamine-HCL stock solution [0.1 % w/v]: 0.10 g/100ml water, then filter sterilize.

Method
Dissolve first 8 components in 800 ml water. Add 200 ml 10 % molasses solution. After autoclaving, cool to ≤ 50 °C before adding 5 ml of 0.1 % thiamine-HCL stock solution. Final pH will be ~7.0±0.2. Sediment in media before and after autoclaving is normal.
Chemical sources:
Na_2HPO_4 (BDH); KH_2PO_4, (JT Baker); NH_4Cl (JT Baker); NaCl (BDH); $MgSO_4 7H_2O$ (BDH); $CaCl_2$ (BDH); $ZnSO_4 7H_2O$ (Fisher); Thiamine-HCl (Fisher); Sucrose (table sugar, Rogers domestic); Molasses (Blackstrap, Crosby's domestic).

8.3 Appendix C: BIOLOG™ GN plate template

Table 8.1. The template for the carbon sources of the BIOLOG™ GN plate.

	1	2	3
A	water	alpha cyclodextrin	dextrin
B	erythritol	D-fructose	L-fucose
C	D-melibiose	b-methyl-D-glucoside	D-psicose
D	acetic acid	*cis*-aconitic acid	citric acid
E	p-hydroxyphenylacetic acid	itaconic acid	a-keto butyric acid
F	bromosuccinic acid	succinamic acid	glucuronamide
G	L-histidine	hydroxy-L-proline	L-leucine
H	urocanic acid	inosine	uridine
	4	5	6
A	glycogen	Tween 40	Tween 80
B	D-galactose	gentobiose	a-D-glucose
C	D-raffinose	L-rhamnose	D-sorbitol
D	formic acid	D-galactonic acid lactone	D-galacturonic acid
E	a-keto glutaric acid	a-keto valeric acid	DL-lactic acid
F	alaninamide	D-alanine	L-alanine
G	L-ornithine	L-phenylalanine	L-proline
H	thymidine	phenylethylamine	putrescine
	7	8	9
A	N-acetyl-D-galactosamine	N-acetyl-D-glucosamine	adonital
B	m-inositol	a-D-lactose	lactulose
C	sucrose;	D-trehalose	turanose
D	D-gluconic acid	D-glucosaminic acid	D-glucoronic acid
E	malonic acid	propionic acid	quinic acid
F	L-ananyl-glycine	L-asparagine	L-aspartic acid
G	L-pyroglutamic acid	D-serine	L-serine
H	2-aminoethanol	2,3-butanediol	Glycerol
	10	11	12
A	L-arabinose	D-arabitol	D-cellobiose
B	maltose	D-mannitol	D-mannose
C	xylitol	pyruvic acid methyl ester	succinic acid mono-methyl-ester
D	a-hydroxybutyric acid	b-hydroxybutyric acid	sigma-hydroxybutyric acid
E	D-saccaric acid	sebacic acid	succinic acid
F	L-glutamic acid	glycyl-L-aspartic acid	glycyl-L-glutamic acid
G	L-threonine	D,L-carnitine	sigma-amino butyric acid
H	D,L-a-glycerol phosphate	a-D-glucose-1-phosphate	D-glucose-6-phosphate

8.4 Appendix D: Carbon utilization profiles from 95 carbon sources

The following carbon sources were not utilized by *P. fluorescens* BRG100:

A1- water (negative control)
A2- α-cyclodextrin
A3- dextrin
A4- glycogen
A7- N-acetyl-D-glucosamine
A12- D-cellobiose
C1- D-melibiose
C2- β-methyl-D-glucoside
C3- D-psicose
C4- D-raffinose
C5- L-rhamnose
C9- turanose
D12- Σ-hydroxy butyric acid
E11- sebacic acid
F2- succinamic acid
F11- glycyl-L-aspartic acid
H4- thymidine
H5- phenylethylamine
H8- 2,3-butanediol
H11- α-D-glucose-1-phosphate
H12- D-glucose-6-phosphate

Isolate 1: BRG*gfp*-1

BRG*gfp*-1 can utilize all of the Carbon sources the wild-type can except:
A8- N-acetyl-D-glucosamine
A11- D-arabitol
C8- D-trehalose
D8- D-glucosaminic acid
E8- proprionic acid
E12- succinic acid
F8- L-asparagine
F12- glycyl-L-glutamic acid
G8- D-serine
H10- D, L-α-glycerol phosphate

Isolate 2: BRG*gfp*-2

BRG*gfp*-2 can utilize all of the following Carbon sources that the wild-type can except:
A9- adonitol
B12- D-mannose
C12- succinic acid mono-methyl-ester
D9- D-glucoronic acid
D10- α-hydroxy butyric acid
E5- α-keto valeric acid
E10- D-saccaric acid
E12- succinic acid
F10- L-glutamic acid
G10- L-threonine
H9- glycerol
H10- D,L-α-glycerol phosphate

It can utilize the additional Carbon sources:
C1- D-melibiose

Isolate 3 :BRGgfp-3

BRG*gfp*-3 can utilize all of the following Carbon sources that the wild-type can except:
D4- formic acid
E3- α-keto butyric acid
E4- α-keto glutaric acid
E5- α-keto valeric acid
E6- D, L-lactic acid
F3- glucoronamide
F4- alaninamide
G4- L-ornithine
H3- uridine
H10- D,L-α-glycerol phosphate

It can utilize the additional Carbon sources:
F11- glycyl-L-aspartic acid

Isolate 4: BRG*gfp*-4

BRG*gfp*-4 can utilize all of the following Carbon sources that the wild-type can except:
A5- Tween 40
A9- adonitol
D4- formic acid
D5- D-galactonic acid lactone
D7- D-gluconic acid

D8- D-glucosaminic acid
D9- D-glucoronic acid
D10- α-hydroxy butyric acid
E5- α-keto valeric acid
E8- proprionic acid
E9- quinic acid
F3- glucoronamide
F4- alaninamide
F5- D-alanine
F9- L-aspartic acid
F12- glycyl-L-glutamic acid
G8- D-serine
G9- L-serine
H9- glycerol
H10- D,L-α-glycerol phosphate

It can utilize the additional Carbon sources:
D12- Σ -hydrobutyric acid

Isolate 5: BRG*gfp*-5

BRG*gfp*-5 can utilize all of the following Carbon sources that the wild-type can except:
C7- sucrose

Isolate 6: BRG*gfp*-6

BRG*gfp*-6 can utilize all of the following Carbon sources that the wild-type can except:
D1- acetic acid
D10- α-hydroxy butyric acid
E5- α-keto valeric acid
E7- malonic acid
E8- proprionic acid
F3- glucoronamide
F4- alaninamide
F12- glycyl-L-glutamic acid
G8- D-serine
G11- D, L-carnitine

Isolate 7: BRG*gfp*-7

BRG*gfp*-7 can utilize all of the following Carbon sources that the wild-type can except:
A8- N-acetyl-D-glucosamine
A9- adonitol
C8- D-trehalose

H9- glycerol
H10- D,L-α-glycerol phosphate

It can utilize the additional Carbon sources:
H12- D-glucose-6-phosphate

Isolate 8: BRG*gfp*-8

BRG*gfp*-8 can utilize all of the following Carbon sources that the wild-type can except:
E5- α-keto valeric acid

Isolate 9: BRG*gfp*-9

BRG*gfp*-9 can utilize all of the following Carbon sources that the wild-type can except:
B7- m-inositol
E5- α-keto valeric acid
E9- quinic acid
F3- glucoronamide
F4- alaninamide
F12- glycyl-L-glutamic acid
H10- D,L-α-glycerol phosphate

Isolate 10: BRG*gfp*-10

BRG*gfp*-10 can utilize all of the following Carbon sources that the wild-type can except:
A9- adonitol
A10- L-arabinose
D4- formic acid
E5- α-keto valeric acid
F3- glucoronamide
F4- alaninamide

Isolate 11: BRG*gfp*-11

BRG*gfp*-11 can utilize all of the following Carbon sources that the wild-type can except:
E3- α-keto butyric acid
E5- α-keto valeric acid
F3- glucoronamide
G8- D-serine
H3- uridine

Isolate 12: BRG*gfp*-12

BRG*gfp*-12 can utilize all of the following Carbon sources that the wild-type can except:
E5- α-keto valeric acid

Isolate 13: BRG*gfp*-13

BRG*gfp*-13 can utilize all of the following Carbon sources that the wild-type can except:
A5- Tween 40
B1- erythritol
B2- D-fructose
B4- D-galactose
B6- α-D-glucose
D1- acetic acid
D4- formic acid
E5- α-keto valeric acid
F1- bromosuccinic acid
F3- glucoronamide
G1- L-histidine
G8- D-serine
H3- uridine

Isolate 14: BRG*gfp*-14

BRG*gfp*-14 can utilize all of the following Carbon sources that the wild-type can except:
E5- α-keto valeric acid

Additional C sources:
F2- succinamic acid

Isolate 15: BRG*gfp*-15

BRG*gfp*-15 can utilize all of the following Carbon sources that the wild-type can except:
B8- α-D-lactose
E5- α-keto valeric acid

Isolate 16: BRG*gfp*-16

BRG*gfp*-16 can utilize all of the following Carbon sources that the wild-type can except:
C7- sucrose
E6- D, L-lactic acid

Additional C sources:
F2- succinamic acid

8.5 Appendix E: Countable range with CIA-BEN

Spiral Plate method countable range using 50 ul or 100 ul deposition volume					
Dilution	**Countable range**			**Countable range**	
Level	**50 ul deposition volume**			**100 ul deposition volume**	
Plated	Min	Max		Min	Max
(10X - DF)	Count	Count		Count	Count
	Cells/ml or g.			Cells/ml or g.	
0	1.60E+03	3.29E+04		8.00E+02	1.65E+04
1	1.60E+04	3.29E+05		8.00E+03	1.65E+05
2	1.60E+05	3.29E+06		8.00E+04	1.65E+06
3	1.60E+06	3.29E+07		8.00E+05	1.65E+07
4	1.60E+07	3.29E+08		8.00E+06	1.65E+08
5	1.60E+08	3.29E+09		8.00E+07	1.65E+09
6	1.60E+09	3.29E+10		8.00E+08	1.65E+10
	Cells/ml Log10			Cells/ml Log10	
DF	Min	Max		Min	Max
0	3.20	4.52		2.90	4.22
1	4.20	5.52		3.90	5.22
2	5.20	6.52		4.90	6.22
3	6.20	7.52		5.90	7.22
4	7.20	8.52		6.90	8.22
5	8.20	9.52		7.90	9.22
6	9.20	10.52		8.90	10.22

8.6 Appendix F: Recipe for phosphate buffer:

Ingredients:

Stock solution A (NaH_2PO_4 monobasic) 31.2 g/litre water
Stock solution B (Na_2HPO_4 dibasic) 53.65 g/litre water

In 1 litre volumetric flasks, prepare the above solutions.

Mix together 195 ml of Solution A and with 305 ml of solution B and 500 ml distilled

water; label as “Phosphate buffer”.

8.7 Appendix G: Observation of green foxtail seedlings under the epifluorescent microscope (with liquid broth)

Growth pouch 1:

Seedling 1:
-bacteria are spread evenly over proximal 1/3 section of root; some microcolonies, some single cells.
-high preference for root hairs
-amount of bacteria appear to increase on the middle 1/3 section of the root
-very low amount on distal 1/3 section.

Seedling 2:
-spread evenly over proximal 1/3 section, some microcolonies
-high amount on root hairs
-less on middle 1/3 section than proximal section
-even less on distal 1/3 section
-clearly highest numbers on proximal 1/3 section of root.

Seedling 3:
-spread evenly over proximal 1/3 section, some microcolonies
-high amount on root hairs
-less on middle 1/3 section than proximal section
-heavy colonization of distal 1/3 section, large microcolonies

Growth pouch 2:

Seedling 1:
-spread evenly over proximal 1/3 section, some microcolonies
-high amount on root hairs
-less on middle 1/3 section than proximal section
-less than middle 1/3 section spread along distal 1/3 section, however heavily colonized at root tip

Seedling 2:
-spread evenly over proximal 1/3 section, some microcolonies
-high amount on root hairs
-less on middle 1/3 section than proximal section
- less than middle 1/3 section spread along distal 1/3 section, however heavily colonized at root tip, large microcolonies

Seedling 3:
-evenly spread throughout proximal and middle sections
- root hairs heavily colonized
-largest amount on distal section

Growth pouch 3:

Seedling 1:
-spread evenly throughout length
-root hairs heavily colonized

Seedling 2:
-spread evenly along proximal 1/3 section
-microcolonies continuously down the length of the root
-root film along length
-appears the bacteria are trapped in some type of mucilaginous film
-root hairs heavily colonized

Seedling 3:
-microcolonies spread evenly throughout length of root
-root hairs heavily colonized

Growth pouch 4:

Seedling1:
-root hairs heavily colonized
-microcolonies spread evenly throughout length of root, however largest amount on proximal 1/3 section

Seedling 2:
-root hairs heavily colonized
-microcolonies spread evenly throughout length of root, however largest amount on proximal 1/3 section

Seedling 3:
-root hairs heavily colonized
-microcolonies spread evenly throughout length of root, however largest amount on proximal 1/3 section

Growth pouch 5:

Seedling 1:
-microcolonies along distal section
-root growing off of main root
-microcolonies spread evenly throughout length of root, all 3 sections were heavily colonized
-root hairs heavily colonized

Seedling 2:
-microcolonies along distal section
-root growing off of main root
-microcolonies spread evenly throughout length of root, all 3 sections were heavily colonized
-root hairs heavily colonized

Seedling 3:
- heavy colonization of root hairs; bacteria appear to be encased in mucilage at very end of root
-microcolonies spread evenly throughout length of root, all 3 sections were heavily colonized

Growth pouch 6:

Seedling 1:
-microcolonies spread evenly throughout length of root, all 3 sections were heavily colonized
-root hairs heavily colonized
-heavy colonization at tip of secondary root

Seedling2:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section, least on distal section
-root hairs heavily colonized

Seedling 3:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section, least on distal section
-root hairs heavily colonized

Growth pouch 7:

Seedling 1:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section, least on distal section
-root hairs heavily colonized

Seedling 2:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section
-least on distal section, although lots of microcolonies at root tip
-root hairs heavily colonized

Seedling 3:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section, least on distal section
-root hairs heavily colonized

Growth pouch 8:

Seedling 1:
- heavy colonization of root hairs; bacteria appear to be encased in mucilage at very end of root
-microcolonies spread evenly throughout length of root, all 3 sections were heavily colonized

Seedlings 2:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section, least on distal section
-root hairs heavily colonized

Seedling 3:
-microcolonies spread evenly throughout length of root
-heaviest colonization on proximal section, followed by middle section, least on distal section
-root hairs heavily colonized

Growth pouch 9:

Note: No fluorescence visible on any seedlings, possibly because this growth pouch was very dried out.

8.8 Appendix H: Observation of green foxtail seedlings under the epifluorescent microscope (with pesta granules)

Growth pouch 1:

Seedling 1:
-bacteria still encased in pesta granules
-most colonization on proximal section
-still a fair amount on middle section
-very little on distal section

Seedling 2:
-bacteria still encased in pesta granules
-most colonization on proximal section
-still a fair amount on middle section
-very little on distal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-heavy colonization near root tip, but not along rest of distal section

Growth pouch 2:

Seedling 1:
-all colonization on proximal section

Seedling 2:
-all colonization on proximal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-heavy colonization near root tip, but not along rest of distal section

Growth pouch 3:

Seedling 1:
-very little fluorescence visible on any of the sections

Seedling 2:
-bacteria still encased in pesta granules
-most colonization on proximal section
-still a fair amount on middle section
-very little on distal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-still a fair amount on middle section
-very little on distal section

Growth pouch 4:

Seedling 1:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-heavy colonization near root tip, but not along rest of distal section

Seedling 2:
-bacteria still encased in pesta granules
-most colonization on upper section
-still a fair amount on middle section, but less than upper
-heavy colonization near root tip, but not along rest of distal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-heavy colonization near root tip, but not along rest of distal section

Growth pouch 5:

Seedling 1:
-very little fluorescence visible on any of the sections

Seedling 2:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-very little on distal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-heavy colonization near root tip, but not along rest of distal section

Growth pouch 6:

Seedling 1:
-all colonization on proximal section

Seedling 2:
-all colonization on proximal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-very little on distal section

Growth pouch 7:

Seedling 1:
-very little fluorescence visible on any of the sections

Seedling 2:
-very little fluorescence visible on any of the sections

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-still a fair amount on middle section, but not as much as upper
-very little on distal section

Growth pouch 8:

Seedling 1:
-all colonization on proximal section

Seedling 2:
-all colonization on proximal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-still a fair amount on middle section, but not as much as upper
-very little on distal section

Growth pouch 9:

Seedling 1:
-very little fluorescence visible on any of the sections

Seedling 2:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-very little on distal section

Seedling 3:
-bacteria still encased in pesta granules
-most colonization on proximal section
-slight colonization on middle section
-heavy colonization near root tip, but not along rest of distal section

8.9 Appendix I: Redi Earth

Ingredients:

4 55-65% Canadian Sphagnum Peat Moss
4 Horticultural grade Vermiculite
4 Starter Nutrient Charge
4 Dolomitic Limestone and a Wetting Agent

Produced by Sun Grow Horticulture